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THE ACETYLCHOLINE SENSITIVITY OF FROG MUSCLE FIBRES AFTER COMPLETE OR PARTIAL DENERVATION

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Structures deprived of their innervation become supersensitive to their normal chemical transmitter as well as to other substances (see Cannon & Rosenblueth, 1949).

Nerve cells usually have a great number of fibres converging upon them and are 'innervated' at numerous points of their surface (Wyckoff & Young 1956). Skeletal muscle fibres may also be innervated at more than one point (Katz & Kuffler, 1941; Hunt & Kuffler, 1954), and slow muscle fibres may have many synapses distributed along their length (Couteaux, 1955). This morphological feature immediately prompts the question as to the effects of partial denervation of these tissues and how they compare with those which follow complete denervation.

METHODS

Material and preliminary operations. Individual muscle fibres in the frog's sartorius are known to have two or more end-plates (Katz & Kuffler, 1941; Hunt & Kuffler, 1954; Iwasaki, 1957) which makes this muscle particularly suitable for these experiments. Frogs (*Rana temporaria*) were anaesthetized by immersion in a solution of 5% ether in water. The skin of the ventral part of the left leg was incised longitudinally from the knee up to near the symphysis. The sartorius was freed along its lateral border for most of its length, and retracted to the medial side in order to expose fully the muscle's deep surface. With suitable illumination and a dissecting microscope (20×) the pattern of innervation could be easily seen; although there is some variation it usually conforms to the type shown in Fig. 4.

The tibial or either one or both of the pelvic nerve branches were cut and removed as far distally as possible, in order to delay regeneration. In the initial experiments the tibial branch was cut, but it was soon found that regeneration occurred more readily than with the pelvic branches, so these were the ones preferentially divided in the later experiments. A drawing was made of the innervation pattern and of the nerve branches cut at the time of operation. The skin was sutured with 4–5 stitches and each frog was labelled by incising interdigital membranes in the hind feet.

For complete denervation the sciatic nerve was cut in the pelvis (just rostral to the pyriformis and vastus externus muscles), and 4–5 mm of it was removed, up to the crural branch which was left intact. A second operation was done one or two months later whenever long denervation periods were desired. This consisted in dividing the 7th, 8th, 9th spinal nerves from their emergence from the spinal canal down to the site of the first section. Groups of about seven frogs were kept without feeding in glass jars in shallow water at room temperature (13–25° C); the water was changed about every third day. Out of

thirty-two frogs with partial denervation only two died (1 and 3 days after the operation). The experiments were made between August 1958 and May 1959.

At variable intervals after the operation paired sartorius muscles were dissected and mounted side by side in a chamber containing 15–20 ml. of Ringer's solution (mM: NaCl 116, KCl 2, $CaCl_2$ 1·8). The nerves lay in separate moist compartments on pairs of Pt electrodes connected to the same stimulator through a transformer. In some cases a small single chamber of 3 ml. capacity was used.

Solutions were run through the muscle chamber by means of two tubes connected to flasks, one containing normal Ringer's solution and the other the test solution. The rate of perfusion was not the same in all experiments: it was usually of the order of 10–30 ml./min. The direction of flow was nearly always from tibial to pelvic regions, so that usually the innervated part of the muscle was first reached by the test solution. In some experiments the concentration of acetylcholine (ACh) at which the muscle fibres twitched was determined by rapidly dipping the muscles in beakers containing ACh at different concentrations. Successive tests were made at 2–5 min intervals. In most experiments neostigmine methylsulphate (Roche Products, Ltd), 10^{-6} (w/v) was added to the solutions. When iontophoretic pulses of ACh were applied no prostigmine was used. Curare was D-tubocurarine chloride (Burroughs Wellcome).

Iontophoretic application of ACh. The method was similar to that described by Nastuk (1953) and del Castillo & Katz (1955). Micropipettes were filled with an approximately 4 M solution of AChCl (Roche Products, Ltd). They were connected to a square pulse generator through a 10 MΩ resistor and to a variable d.c. bias through 50 MΩ. ACh pipettes were stored at 6° C for many months without obvious deterioration. After using a pipette its outside was rinsed with distilled water and the pipette stored again for use in further experiments; it was discarded when its tip broke or collected dirt and so became difficult to observe.

Electrical recording and apparatus. Micro-electrodes were filled with 3 M-KCl by the method of Caldwell & Downing (1955). Potentials were recorded after DC amplification. A switch in the leads to one cathode follower made it possible to connect the micro-electrode either to the grid of the input valve or to the output of a square pulse generator while the micro-electrode was inside the fibre. Another switch connected the grid of the cathode follower to the monitor resistance (usually 15 kΩ) whenever the micro-electrode was used for passing current through the fibre.

RESULTS

Completely denervated muscle

ACh sensitivity. **The ACh concentration in the bath needed to evoke a few mV depolarization or a twitch in denervated fibres was from ten to several thousand times lower than that required by normal fibres. This large variation in denervation supersensitivity was noted not only when comparing muscles from different frogs but also when comparing different muscles from the same animal.**

This type of diffuse application of drugs could conceivably produce effects different from those obtained by localized application of the drug. In order to compare in different fibres the reactions of comparatively small regions of the membrane to ACh, the drug was applied by iontophoresis through a micropipette. The standard procedure was to lower the ACh pipette until it just touched the muscle fibre. Slight upwards and downwards displacements were then made until the maximal response to a given 'Coulomb-pulse' was obtained. This process was repeated at different points on a given fibre and a graph of the distribution of

'ACh sensitivity' along its length could then be obtained (Fig. 1). The recording electrode was moved in steps of 1–2 mm when it was desired to explore a length of fibre greater than that.

The sensitivity to ACh iontophoretically applied is expressed as the ratio of the ACh potential (mV) to the nano-coulombs which released the ACh. In evaluating it certain factors had to be considered, e.g. that an S-shaped relation exists between quantity of current (coulombs) passing through the pipette and the depolarization set up by the released ACh. Furthermore, a given number of coulombs produces a greater ACh potential if delivered in a short time than if a longer pulse is used. For these reasons a pulse of 3·7 msec duration was always used, to produce an ACh potential of about 1 mV, when comparing maximal ACh sensitivities of different fibres.

In contrast to the supersensitivity observed with general bath application, the local sensitivity of denervated end-plates to micro-application of ACh does not appear to be significantly higher than that of normal end-plates. The mean ($\pm$ S.E.) of the maximal sensitivity at 15 denervated end-plates (20–111 days after nerve section) was $12{\cdot}8 \pm 3{\cdot}6$ mV/nC, compared with a mean of $8{\cdot}9 \pm 3{\cdot}0$ at eight normal end-plates in the corresponding control muscles.

The extent of ACh-sensitive membrane. A recording micro-electrode was first inserted in a region where miniature end-plate potentials (m.e.p.p.s) could be detected. ACh was then applied iontophoretically at varying distances from the recording electrode. The duration of the current pulse was usually 3·7 msec, except when the local sensitivity was very low, in which case pulse durations had to be increased up to 5 sec. The limits of the ACh-sensitive region of a fibre were taken as those where the sensitivity had fallen to something like 1/10,000 of the maximal.

A normally innervated muscle is sensitive to ACh only in an area restricted to and closely surrounding the neuromuscular junction. The total length of this region varies from fibre to fibre. It averaged about 1·1 mm (0·3–2·0 mm) in 13 sartorius fibres (Fig. 1). At a given distance from the peak position some points on the fibre circumferences are much less sensitive than others, which is to be expected from what is known of the terminal arborization of the nerve fibre (Kühne, 1887). In some fibres one may only obtain a small response to brief ACh pulses, probably because the synaptic regions are situated on the deep surface of the fibres. Cholinesterase is concentrated at both the neuromuscular and the musculotendinous junctions (Couteaux, 1953; Gerebtzoff, 1954) but no responses to iontophoretically applied ACh could be obtained from musculotendinous junctions in normal frog sartorius fibres.

Seven to eight weeks after denervation the length of membrane sensitive to ACh had increased to 5–6 mm. Responses could be obtained from anywhere within the sensitive portion, which included the site of the original end-plate. The latter could be located by the presence of

low-frequency m.e.p.p.s which occur at denervated end-plates (Katz & Miledi, 1959).

One fibre examined 51 days after denervation, although not sensitive at its pelvic end, responded to ACh over approximately 10 mm of its length.

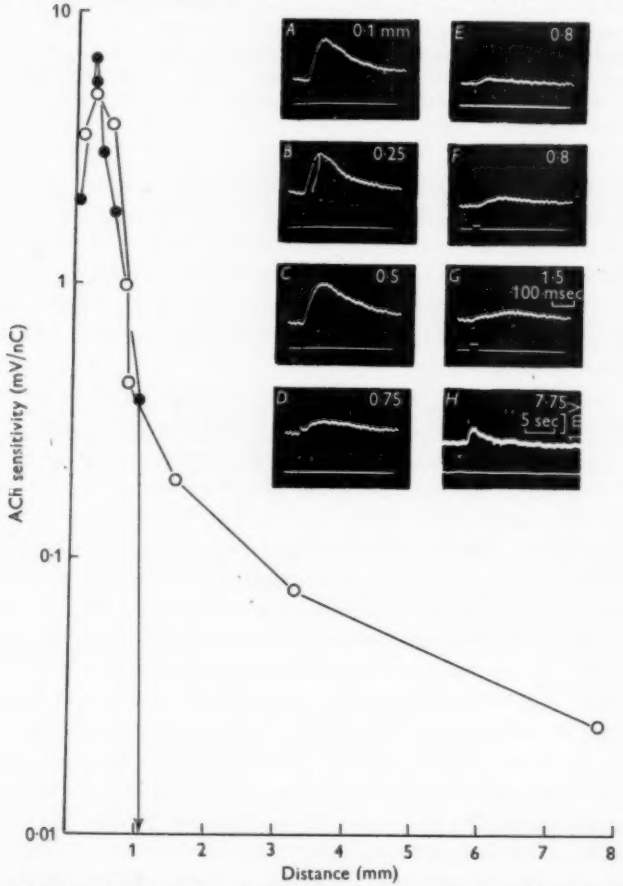

Fig. 1. Distribution of acetylcholine sensitivity in a muscle fibre (resting potential 90 mV) 66 days after complete denervation (○) and at a normal end-plate (resting potential 94 mV) in the control muscle (●). Abscissae, distance along fibre; ordinates, sensitivity to iontophoretic pulses of ACh, log. scale. Inset: upper traces, examples of ACh potentials of denervated fibre at specified distances; lower traces, records of current flowing through acetylcholine pipette. Pulse duration is 3·7 msec in *A–E*, and 37 msec in *F–H*; time base calibration in *G* applies to records *A–G*; voltage and current calibration for all records in *H*. Monitor calibration: vertical bar in $H = 2{\cdot}2 \times 10^{-7}$ A. Muscle supersensitivity, 100. In this and following figures the muscle supersensitivity is expressed as the ratio, control:denervated, of minimal ACh concentrations which evoke muscle twitches.

The distribution of sensitivity showed three peaks; two of them were located at usual end-plate foci while the intermediate lay at a region traversed by a degenerated nerve branch, possibly also an original end-plate position.

The whole length of the muscle became sensitive to ACh some 10 weeks after denervation. This is illustrated in Fig. 1, where for comparison the distribution of sensitivity at an end-plate in the opposite, normal, muscle is also shown. The musculotendinous junction of the denervated fibre was near the position 8 mm. The responses at points beyond 1 mm could not be explained by diffusion of ACh to more sensitive regions, because no response was detected if the ACh pipette was moved transversely 100 μ away from the edge of the fibre. All the other fibres examined in this muscle were sensitive to ACh near the pelvic tendon, nevertheless the sensitivity at the end-plate was still up to 1000 times greater. Not only did the sensitivity decrease with increasing distances from the end-plate but the time course of the potentials evoked by the ACh became slower (Fig. 1 inset; compare Fig. 8).

The ACh sensitivity of the muscle fibres near their pelvic tendon goes on increasing with time after denervation, but even 158 days after nerve section the sensitivity at the end-plate is several hundred times greater. In these cases the muscles had become very atrophic (cf. Birks, Katz & Miledi, 1959). Individual fibres were quite small and hardly recognizable under the dissecting microscope. The relatively greater density of connective tissue made it difficult to insert the micro-electrode and the fibres appeared to have low resting potentials, but this was difficult to ascertain. Twitches could be evoked by applying ACh with the pipette anywhere in the muscle. The amount of ACh required was lowest at the usual end-plate regions.

Denervated fibres which had acquired sensitivity to ACh near their pelvic tendon were examined to see if m.e.p.p.s could be detected there. However it was found that these potentials, caused presumably by small packages of ACh (Katz & Miledi, 1959; Birks, Katz & Miledi, 1960), remained localized to the end-plate region, even though the whole membrane was sensitive to ACh.

The responses to repeated pulses of ACh progressively diminish in amplitude. Regions of the muscle fibres where sensitivity was low were desensitized more readily than those where sensitivity was higher. For example, pulses of ACh were applied at different points of a denervated fibre which was sensitive over 5 mm of its length. The decline in the amplitude of consecutive responses was greater the smaller the initial local sensitivity (Fig. 2). The responses were not only reduced in amplitude but also became slower in time course. Recovery from the desensitization was

usually slower than the onset, and some minutes had to elapse before the response recovered its original amplitude and time course. The rate of recovery was approximately the same, irrespective of the local sensitivity. When ACh was applied again before recovery was complete, further desensitization followed a course similar to the first one (Fig. 2).

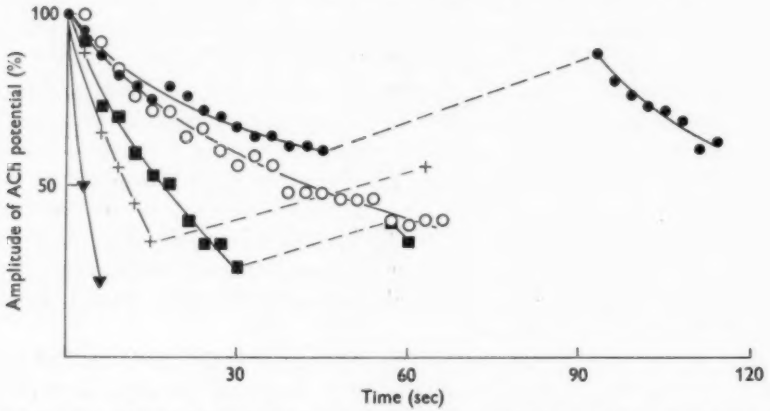

Fig. 2. Desensitization at points with different ACh sensitivities in a fibre 51 days after complete denervation. Abscissae, time after first acetylcholine pulse; ordinates, amplitude of consecutive ACh potentials (as % of first). Sensitivities (mV depolarization/nC) and distances (mm) from point of maximum sensitivity were: (●) 8·7, 0·05; (○) 6·6, 0·5; (■) 2·0, 1·0; (+) 0·9, 1·25; (▼) 0·06, 1·75. Interrupted lines indicate recovery from desensitization. Resting potential 80 mV.

After long denervation periods, when the whole muscle fibre surface had become sensitive to ACh, desensitization was particularly marked near the musculotendinous junction where sensitivity was lowest (Fig. 3). This circumstance made it necessary to avoid any release of ACh until the pipette was well in position, because at the stage at which the muscle fibre membrane is just becoming sensitive to ACh even a single test dose may prevent any further reaction to ACh for several minutes. Nevertheless if after desensitization the ACh pipette is moved to another region of the same fibre, a few hundred microns away, responses are again immediately obtained (Fig. 3).

Prolonged applications of ACh. The supersensitivity and the spread of the chemo-sensitive area of membrane which follow denervation might in some way be caused by the decrease in the amount of ACh which normally impinges on the end-plate. This possibility was tested as follows.

Frog muscles (sartorius, iliofibularis, gastrocnemius, semitendinosus) can be isolated and kept, without any special precaution, in Ringer's solution at 6° C for more than 15 days, and for about 2 days at 22° C. Isolated denervated muscles kept at 6° C remained supersensitive

to ACh for more than 15 days. However, the sensitivity of both the normal and the denervated muscles tended to go down to about 1/10 of its original value during the first 6 days after isolation. The concentration of ACh at which the denervated muscle twitched was about 100 times lower than that for the control muscle during the whole period of observation. At 22° C the sensitivity also tended to decrease in both the denervated and normal muscles, but the former remained more sensitive until they both became inexcitable.

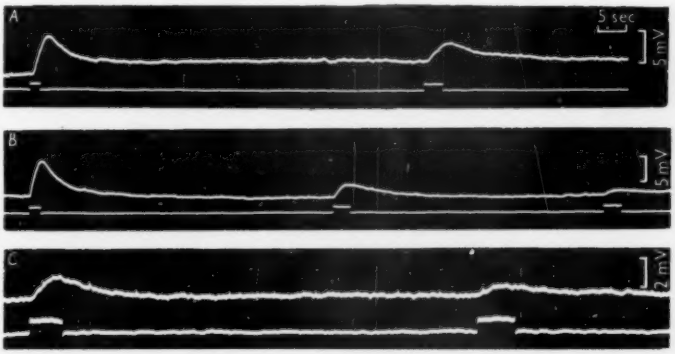

Fig. 3. Responses to iontophoretic pulses of ACh applied near the pelvic tendon of completely denervated sartorius fibres. Upper traces, intracellular records of ACh potentials; lower traces register currents through pipette. *A* and *B*, records from same fibre (89 mV). After the fibre had become desensitized in *A* the pipette was moved 0·5 mm along the fibre and *B* was obtained. *C*, ACh potentials from another fibre (resting potential 90 mV) in same muscle, 88 days after denervation. Time calibration for all records in *A*. Vertical bars are also monitor calibration: *A*, 3×10^{-7} A; *B*, 3×10^{-7} A; *C*, 10^{-8} A.

Normal and denervated muscles were kept at 6° C in 5×10^{-8} ACh in slightly acid (pH about 5·8) Ringer's solution for the first day after isolation. The concentration was then increased to 1×10^{-6} and the muscles were kept in it for 4 more days. The solutions were frequently replaced by fresh ones, and the ACh in the solution to be discarded was often assayed (with one of the denervated muscles kept in normal Ringer's solution) and its concentration found to be not greatly reduced. The sensitivity of both muscles was determined, once or twice a day, approximately 30 min after transferring them to ACh-free Ringer's solution at room temperature. The denervated muscle was always more sensitive than either an innervated muscle also kept in ACh or a control muscle kept in ACh-free solution.

In a similar experiment, a sartorius that had been denervated 158 days previously was kept in 10^{-6} ACh at room temperature for 24 hr. The muscle fibres were known to be sensitive to ACh near their pelvic tendons and gave twitches when ACh was applied through a pipette. While the

muscle was in ACh no twitches could be obtained on iontophoretic application of ACh, but they occurred readily 20 min after washing out the ACh. The pelvic end of the muscle fibre was still sensitive to ACh at the end of the 24 hr experiment.

Partially denervated muscle

Acetylcholine sensitivity. It is clear from what is known about multiple innervation in frog's sartorius (see also Katz & Kuffler, 1941) that most and probably all fibres of the operated muscles had at least one innervated and functional end-plate. The purpose of these experiments was, accordingly, to see whether this residual innervation could prevent the changes occurring at the other end-plate after its nerve supply had been removed. The concentration of ACh at which muscle fibres contracted was determined in twenty partially denervated muscles, and invariably found to be lower than that which caused twitches in the most sensitive fibres of the control. A similar increased sensitivity was obtained in thirteen cases in which ACh solutions were run through the chamber while intracellular potentials were recorded, simultaneously, from a denervated and an innervated end-plate in the same muscle.

More direct are experiments where ACh sensitivity was compared in a denervated and an innervated end-plate in the same muscle. To attain this, a micro-electrode was inserted in a muscle fibre at the region of the denervated end-plate. This micro-electrode was used to apply hyperpolarizing pulses to the fibre and a second micro-electrode was then inserted at different distances until the fibre had been identified by the presence of electrotonic potentials at 5–8 mm from the denervated end-plate. The second electrode was left inserted and now used for applying the hyperpolarizing pulses while a third micro-electrode was placed at progressively increasing distances until it reached an innervated end-plate. Distances between the two end-plate positions located in this way varied between 8 and 15 mm. Only surface fibres were aimed at and some had to be discarded because they were no longer superficial in the region of the innervated end-plate. Furthermore, in some muscles it was difficult to follow the fibres on account of the large amount of fibrous connective tissue which had probably proliferated as a result of the operation.

Figure 4 shows results from an experiment made 15 days after denervation. It is seen that transient exposure of the whole muscle to ACh 10^{-8} produced a depolarization of 7 mV at the denervated end-plate while no depolarization could be detected at the innervated end-plate (Fig. 4*A*). A higher concentration (5×10^{-8}) depolarized the denervated end-plate by 13 mV and the innervated by approximately 0·7 mV (Fig. 4*B*). A still higher concentration applied for a longer time evoked a bigger and longer-

lasting depolarization of 32 mV at the denervated end-plate and still only about 3 mV at the innervated (Fig. 4*C*).

If a sufficiently high concentration was used action potentials were initiated at the denervated end-plate where they could be seen to arise from a slowly increasing local depolarization in contrast to the more abrupt rise of the propagated impulse at the innervated end-plate (Fig. 5).

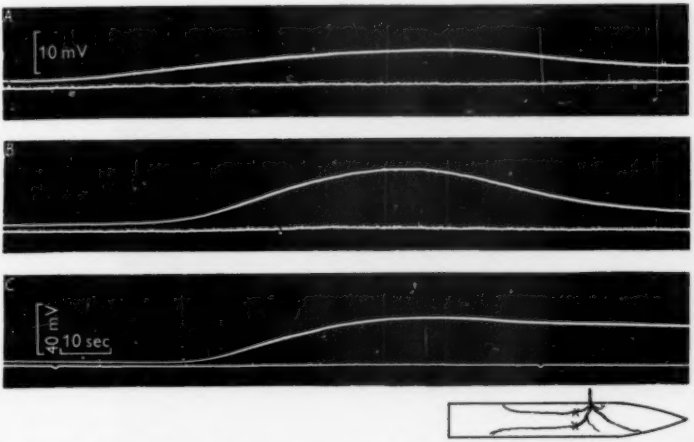

Fig. 4. Effect of diffuse application of ACh to a partially denervated muscle fibre. Simultaneous intracellular records from a denervated (upper traces) and an innervated (lower traces) end-plate in the same muscle fibre, 15 days after section of both pelvic nerve branches. Distance between end-plates, 14·5 mm. Resting potentials were 86 and 84 mV at the denervated and innervated end-plates, respectively. During exposure to ACh: *A*, 10^{-8}; *B*, 5×10^{-8}; *C*, 10^{-7} at 20 min intervals. Voltage calibration same for both traces, *B* as in *A*; Time calibration in *C* applies also to *A*, and *B*. Prostigmine, 10^{-6}. Muscle supersensitivity, 50. The diagram illustrates usual arrangement of nerve branches in frog's sartorius. The crosses show sites where nerves were cut.

The first action potential starts at about 33 mV depolarization, and has an amplitude of 100 mV measured from the resting potential level, but successive spikes arising at the denervated end-plate diminish progressively in size. Although the peak amplitude of spikes at the innervated end-plate was not recorded, it appears from the photographed part of the action potentials that the impulses had grown to more or less their full size by the time they arrive there. The decrease in amplitude of the locally initiated action potentials shown in Fig. 5 is not due to permanent damage such as might be produced by the micro-electrode, for later on a stimulus to the nerve elicited a full-size action potential at this region. In Fig. 6*A* the impulse can be seen to travel from the innervated end-plate at which it

arises to the denervated end-plate with a delay of 9·5 msec, indicating a conduction velocity of 1·5 m/sec for the propagation of the impulse between the two end-plates. Curare prevented the generation of action potentials, and then an end-plate potential could be seen only at the innervated end-plate (Fig. 6*B*). The small potential change at the denervated end-plate is partly due to activity in other fibres.

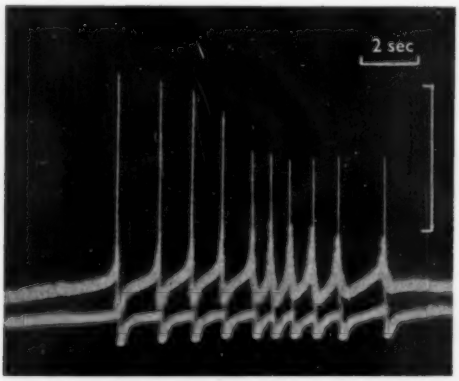

Fig. 5. Intracellular action potentials evoked by slow perfusion with 3×10^{-7} ACh, in same fibre as Fig. 4. Perfusion with Ringer's solution was started just after the first impulses had appeared. Upper trace, denervated end-plate; lower trace, innervated end-plate. Owing to slow film speed, the difference in latency of action potentials is only just perceptible (compare after-hyperpolarizations). Maximal depolarization at innervated end-plate was 12 mV. Voltage calibration: 50 and 20 mV for denervated and innervated end-plates, respectively.

As in completely denervated muscle, the increased ACh sensitivity of partially denervated muscle from different animals was very variable. After partial denervation it ranged from 5 to 5000 times when compared with the muscle from the other leg. The most sensitive muscle encountered had many fibres (resting potentials 80–98 mV) which gave action potentials with an ACh concentration of 2×10^{-10}. By contrast, in two other muscles action potentials were not elicited until an ACh concentration of 3×10^{-7} was reached. The sensitivity of control muscles was also variable, although to a lesser extent: threshold concentrations ranged from 10^{-7} to 5×10^{-6}.

We have seen in the preceding paragraphs that supersensitivity develops in the denervated portions of partially denervated fibres. The sensitivity of the innervated end-plates to diffusely applied ACh was not tested separately. Nevertheless, it does not seem to be very different from that of end-plates in normal muscle, judging by the fact that in twenty fibres from ten different supersensitive muscles bath concentrations of from

10^{-8} to 8×10^{-7} ACh were needed to produce 1–2 mV depolarization at the innervated end-plates. In each case similar or even larger depolarizations could be observed at normal muscle end-plates.

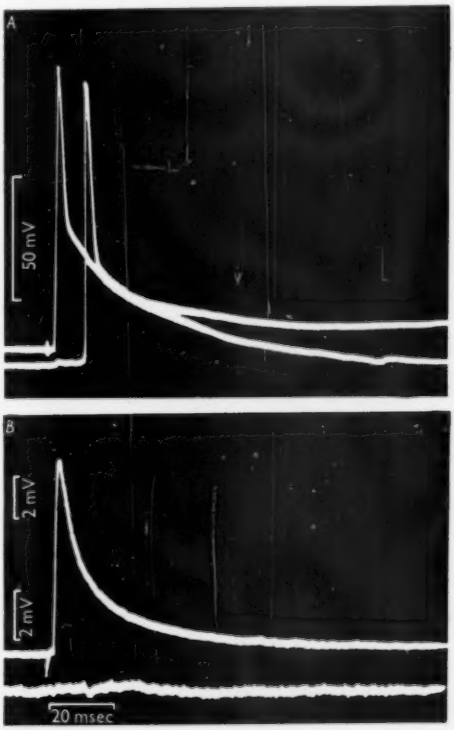

Fig. 6. Intracellular potential changes evoked by nerve stimulation in a partially denervated muscle fibre (same as Fig. 5). Upper traces, records from innervated end-plate; lower traces, records from denervated end-plate. *A*, propagated impulse arising at innervated end-plate; *B*, end-plate potential after adding curare to prevent generation of action potential.

The maximum *localized* sensitivity to brief iontophoretic pulses of ACh at denervated or innervated end-plates in partially denervated fibres was not different from normal. Nevertheless, on testing denervated end-plates 2–3 weeks after nerve section it was immediately noticed that responses to ACh were easily obtained in all fibres examined. The length of fibre sensitive to ACh was increased; it ranged from about 2·3 to 4 mm in four fibres examined. This is illustrated in Fig. 7, obtained from a denervated end-plate 20 days after partial denervation. The recording electrode was first placed at point 0 and the points up to and including

1 mm were tested with the ACh pipette; the recording electrode was then shifted 1 mm and the rest of the results obtained. The length of fibre sensitive to ACh was greater than 2 mm: responses of 0·5 and 2 mV were still obtained at points 0·05 and 2·0 mm. The main difference in the spatial distribution of local sensitivity at this stage of denervation is an extended fringe of relatively low sensitivity.

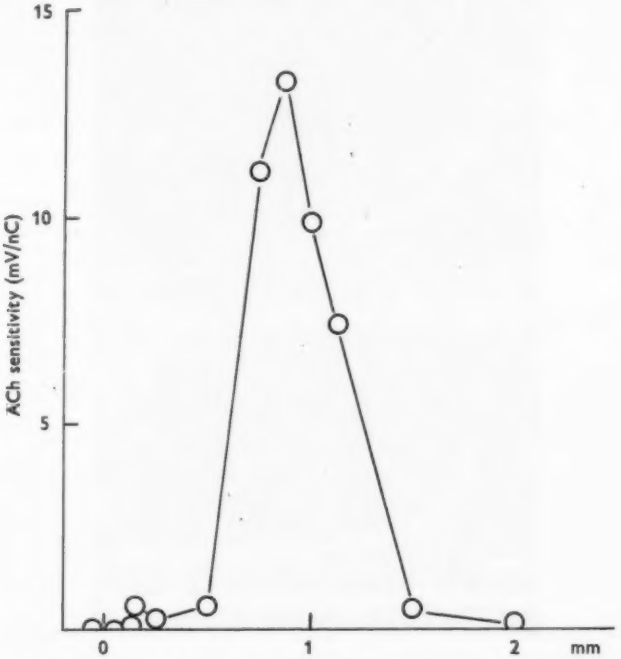

Fig. 7. Distribution of ACh sensitivity at a denervated end-plate, 20 days after partial denervation. Abscissae, distance from point of first insertion of recording micro-electrode; ordinates, ACh sensitivity. Resting potential, 93 mV. Frequency of m.e.p.p.s was 0·02/sec. Muscle supersensitivity, 100.

Some muscles whose pelvic nerve branches had been divided were examined to see if the spread of chemo-sensitive membrane extended as far as the pelvic end of the fibres. There was no indication of this even 73 days after denervation, but at this time all the fibres explored at the end-plate showed some evidence of regeneration.

Just as in completely denervated fibres, repeated application of ACh at spots of initially low sensitivity was followed by a great reduction in the response and a concomitant slowing of its time course. Similar but less pronounced changes occurred at spots of higher sensitivity (Fig. 8).

Spontaneous electrical activity. It has been shown by Katz & Miledi (1959) that after complete denervation of frog muscle fibres the m.e.p.p.s first disappear, only to reappear at a much lower rate a few days later. For the first few days after partial denervation the m.e.p.p.s recorded with a micro-electrode from the denervated regions of the muscle appeared to be

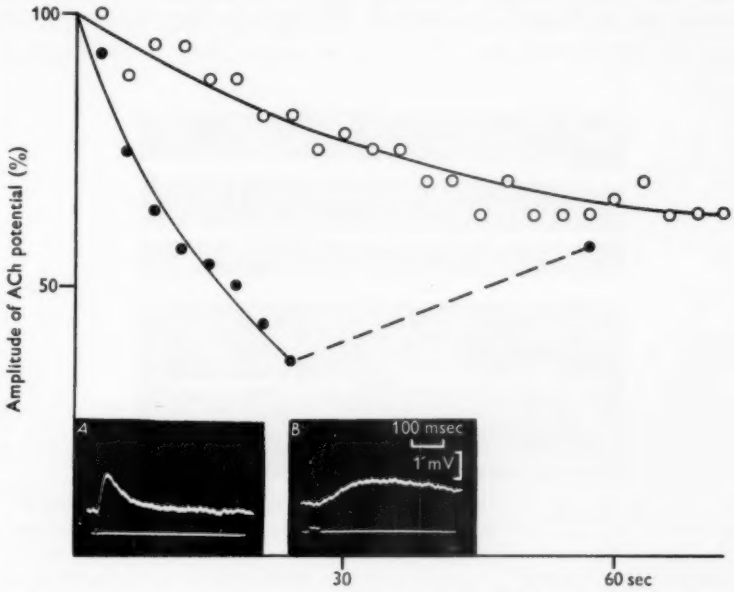

Fig. 8. Course of desensitization at spots with different ACh sensitivity: same end-plate as Fig. 7; (○) and (●) amplitude of consecutive responses to iontophoretic ACh pulses applied respectively to points 0·75 and 0·15 of Fig. 7. Abscissae, time from first stimulus; ordinates, amplitude of ACh potential (as % of first). Interrupted line illustrates recovery from desensitization. The inset shows control ACh potentials at both spots; note the slower time course in the less sensitive spot (*B*); calibration bars apply to both *A* and *B*. Monitor calibration; voltage scale = $2{\cdot}5 \times 10^{-7}$ A.

quite normal both in frequency and amplitude. About 5 days after intramuscular nerve section the m.e.p.p.s disappeared from the denervated end-plates. A few days later m.e.p.p.s could again be recorded from them, but the frequency was on the average fifty times lower than at the innervated end-plates. The amplitudes of the potentials did not differ significantly. This sequence of events is clearly similar to what has been observed in completely denervated muscles (Katz & Miledi, 1959). Not only was the frequency of m.e.p.p.s much lower at the denervated end-plates but their amplitude distribution, as in completely denervated

muscle (cf. Birks *et al.* 1960) was highly skewed in contrast to the normal distribution of amplitudes at the innervated end-plates. The m.e.p.p.s recorded from the innervated portions of partially denervated muscles did not differ in any obvious way from those of control muscles.

The results are best illustrated from experiments where simultaneous records were obtained from two end-plates in the same fibre. Figure 9*A* illustrates the different rates at which m.e.p.p.s appeared in a denervated and an innervated end-plate of the same muscle fibre: 0·027 and 2/sec,

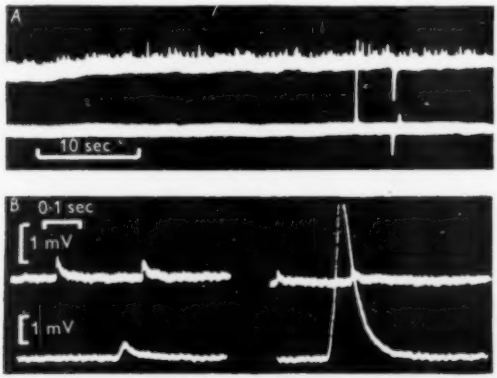

Fig. 9. Simultaneous intracellular records of miniature end-plate potentials at an innervated (upper traces) and a denervated (lower traces) end-plate in the same muscle fibre; same fibre as in Fig. 4 but 2 hr earlier. The downward stroke appearing simultaneously in both traces in *A* corresponds to a hyperpolarizing pulse applied through a third intracellular micro-electrode located 7 mm from the innervated end-plate. Voltage calibrations in *A* same as in *B*.

respectively. Sample potentials are seen in Fig. 9*B*, while their amplitude distribution is shown in Fig. 10. There was no consistent difference in the mean amplitude of the m.e.p.p.s at different end-plates in the same fibre. In seven experiments the mean amplitude (after prostigmine) of m.e.p.p.s at denervated end-plates was $0{\cdot}36 \pm 0{\cdot}04$ mV (mean $\pm$ S.E.), while that of innervated end-plates was $0{\cdot}42 \pm 0{\cdot}07$ mV.

Membrane resistance. Since the membrane resistance of completely denervated muscle fibres is increased (Nicholls, 1956) experiments were made to see if a similar change occurred in partially denervated fibres. The resistance of the muscle fibre membrane was determined with two micro-electrodes, 50–200 μ apart, in the same fibre. One was used for passing currents through the membrane while the other recorded the concomitant change in membrane potential. A current–voltage relation was obtained the slope of which gives the 'input' resistance (R) from which the

specific membrane resistance (R_m) can be calculated. $R_m = \pi^2 R^2 d^3 / R_1$, where d is the fibre diameter and R_1 the specific intracellular resistance in ohm.cm (Katz & Thesleff, 1957).

The input resistance varies greatly from fibre to fibre, largely because of the variable fibre size (cf. Katz & Thesleff, 1957). This variation was greater when comparing input resistance at denervated end-plates. The membrane resistance of partially denervated muscle fibres appeared

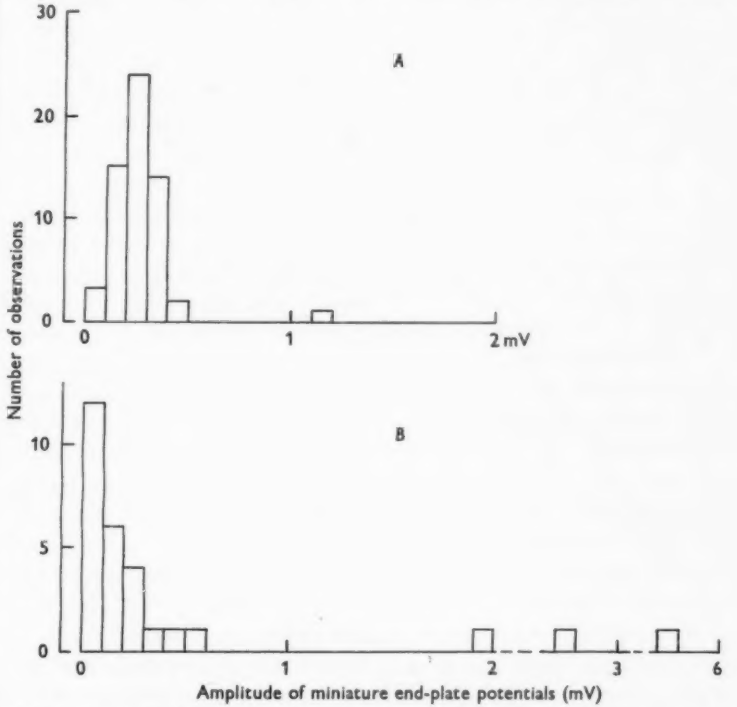

Fig. 10. Amplitude distribution of miniature end-plate potentials at an innervated (*A*) and a denervated (*B*) end-plate in the same muscle fibre; same end-plates as in Fig. 9.

higher at the denervated end-plates than elsewhere. For instance, when compared in the same muscle the input resistance was $4{\cdot}67 \pm 0{\cdot}65 \times 10^5\ \Omega$ at nine denervated end-plates, while at the corresponding innervated end-plates it was $3{\cdot}02 \pm 0{\cdot}23 \times 10^5\ \Omega$. The difference was significant at the 2–3 % level (Student's t test). In another muscle resistances were compared in corresponding regions, at the denervated end-plates, at 4 mm from the pelvic end of the muscle and at the innervated end-plates. The values obtained (in $10^5\ \Omega$) were $10{\cdot}9 \pm 2{\cdot}3$, $6{\cdot}5 \pm 0{\cdot}5$ and $8{\cdot}6 \pm 1{\cdot}1$ respectively.

In two cases input resistance measurements were obtained from two end-plates in the same fibre. Here again the resistance was greater at the denervated than at the innervated end-plate (Fig. 11).

Effect of nerve impulses. It was desirable to find out whether the supersensitivity which follows partial denervation causes changes in the transmission of impulses through the remaining innervated end-plates. First, the paralysing concentration of curare was determined on a muscle of

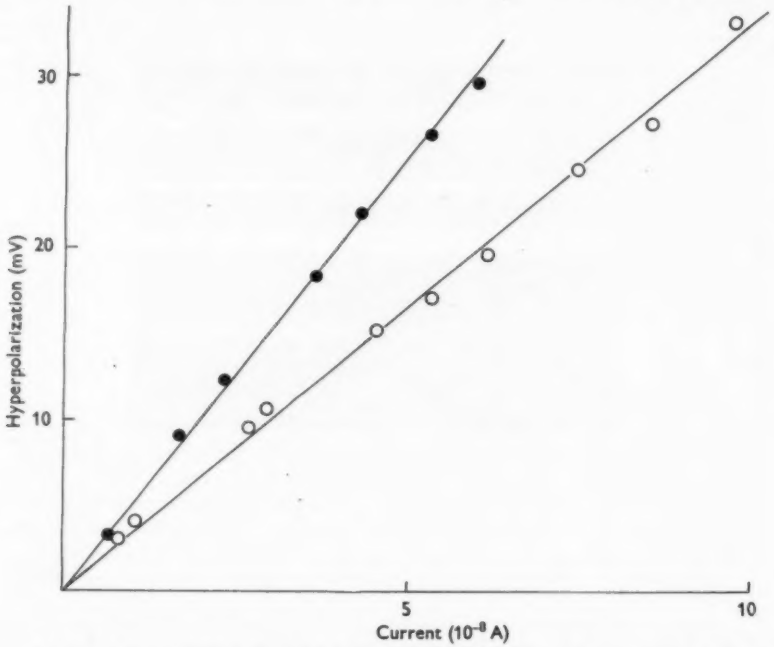

Fig. 11. Current–voltage relation at an innervated (○) and a denervated (●) end-plate of a muscle fibre 34 days after partial denervation. Resting potentials 80 and 84 mV, respectively. Distance between end-plates, 11·7 mm; muscle supersensitivity, 5000.

which only one half-width had been partially denervated. The denervated region of this half was supersensitive to ACh. Nevertheless, the same concentration of curare was required to abolish the indirect response of either half as well as that of the control muscle. Similar results were obtained in another muscle 50 days after partial denervation. In this case the sensitivity of the denervated muscle to diffusely applied ACh was 100 times greater than that of the control muscle. Once the muscles were paralysed end-plate potentials were recorded (in tubocurarine 4×10^{-6}) at fourteen innervated end-plates of the partially denervated muscle and

at the same number of end-plates in the control muscle. The respective mean amplitudes were $4{\cdot}4 \pm 0{\cdot}8$ and $6{\cdot}8 \pm 1{\cdot}8$ mV. That is to say the transmitter substance liberated by the nerve impulse does not exert a supernormal effect on muscle fibres which have a remote denervated portion.

DISCUSSION

The changes which occurred at denervated end-plates in partially denervated fibres were essentially similar to those at end-plates in completely denervated muscle. One difference was that partially denervated muscles did not show atrophy; but regeneration would have to be avoided and longer periods of denervation studied before one can safely conclude that the existence of one functional end-plate in the fibre can permanently prevent denervation atrophy.

The evidence presented shows unequivocally that muscle fibres, although retaining a zone of normal innervation, become supersensitive to diffusely applied ACh in the denervated part. An increased sensitivity of partially denervated nerve cells was postulated by Cannon & Haimovici (1939) who found an increased response of cat motoneurones to injections of strychnine or ACh after previous semisection of the spinal cord.

It is clear that the main factor causing the increased sensitivity of partially or completely denervated muscle to diffusely applied ACh is an increase in the chemo-sensitive membrane area. This property spreads from the end-plate towards the tendon ends and, after complete denervation, eventually involves the whole muscle fibre. Evidence for an increase in the area sensitive to ACh has recently been obtained by Axelsson & Thesleff (1959) in mammalian muscle and by Miledi (1959) in frog muscle. This was also very probably the case in experiments of Kuffler (1943), who mentions that some frog fibres isolated from denervated muscle appeared to be sensitive everywhere, and in experiments of Perry & Zaimis (see Zaimis, 1954) who measured the loss of potassium in decamethonium-treated gastrocnemii of the hen. Other denervation phenomena such as changes in cholinesterase concentration, changes in membrane permeability, muscle atrophy or exposure of the receptors due to degeneration of the nerve terminals can at most only play a minor part in causing supersensitivity.

The end-plate receptors themselves do not appear to be supersensitive to ACh after denervation. This conclusion is based on the fact that the m.e.p.p.s of denervated muscle do not become much greater in amplitude at the time when supersensitivity develops; and that denervated end-plates do not become significantly supersensitive to iontophoretically applied ACh.

On the other hand, receptors which appear after denervation along normally insensitive portions of the membrane might initially be less reactive than those at the end-plate. This could conceivably explain the low sensitivity, and also the slower time course of a given ACh potential. The increase in sensitivity as the time of denervation progresses would then be due to an actual increase in the reactivity of the receptors. But perhaps a more probable explanation would be that each receptor unit is fully reactive at the time of its first appearance, and that sensitivity is simply an index of the local density of receptor units.

Section of the motor nerve is not a necessary condition for the spread in the chemo-sensitive membrane properties. Cannon & Haimovici (1939) reported increased sensitivity of cat striated muscle to intra-arterial injections of ACh after partial denervation of motoneurones; a phenomenon somewhat analogous to the well known supersensitivity which follows 'decentralization', i.e. section of penultimate neurones to effector cells (see Cannon & Rosenblueth, 1949). This increased sensitivity may also be due to an increase in the number of available receptors (cf. Brown, Davies & Ferry, 1959).

An increase in the membrane resistance of frog denervated muscle has been previously described by Nicholls (1956). In partially denervated fibres the increase in membrane resistance is restricted to the denervated end-plate region. This may also be the case in completely denervated fibres, as it actually seemed to be in a preliminary experiment in which this was tested. The cause of the increased resistance remains unknown. Harris & Nicholls (1956) found a decrease in K permeability in denervated muscles; but reduced permeability to Cl ions as well may have to be invoked to explain the slightly greater than twofold increase in membrane resistance. Conceivably membrane sites normally available for these ions are taken up by the increasing 'ACh receptors'.

Katz & Thesleff (1957) have shown that the amplitude of the m.e.p.p.s is proportional to the input resistance of the muscle fibre. Since this is increased at denervated end-plates while the mean amplitudes of m.e.p.p.s at denervated and innervated end-plates in the same fibre are not greatly different, it would appear that the mean size of ACh quanta responsible for the m.e.p.p.s is smaller at the denervated end-plates.

Langley (1916) has shown that the contraction of a muscle becomes slower after denervation. Since the membrane of partially denervated muscle fibres has different properties in its innervated and denervated regions, it is conceivable that there may also be local differences in the mechanical responses of these two regions.

The reaction of supersensitive structures to nerve impulses. The present experimental results have a significant bearing on the problem of trans-

mission of nerve impulses through partially denervated structures. Thus, Simeone, Cannon & Rosenblueth (1938) found that in partially denervated sympathetic ganglia preganglionic nerve stimulation resulted in a greater than normal response of post-ganglionic nerve fibres. A similar increased response of partially denervated structures to nerve impulses is also found in the spinal motoneurones (Bremer, 1928), adrenal gland (Simeone, 1938*a*) and skeletal muscle fibres (van Harreveld, 1945; Hines, Wehrmacher & Thomson, 1945).

It has been suggested that the enhanced action of nerve impulses on incompletely denervated nerve cells is due to an increased sensitivity to the transmitter substance (Cannon & Rosenblueth, 1949). This possibility does not seem very likely in view of the fact that m.e.p.p.s and localized ACh potentials at denervated end-plates were not greater than normal. A more probable explanation is that the denervated synaptic regions become innervated by collaterals arising from the remaining normal fibres. These will therefore elicit a supernormal response. Evidence for such reinnervation by 'sprouting' from normal nerve fibres has been obtained on muscle (Edds, 1950; Hoffman, 1950; van Harreveld, 1945, 1952) and on nerve cells (Murray & Thompson, 1957; Liu & Chambers 1958).

On the other hand, the greater efficacy of nerve impulses on partly denervated or decentralized smooth muscle cells or glands (Cannon & Rosenblueth, 1949) may well be accounted for by a supersensitivity to the transmitter substance, possibly due to a proliferation of receptors. In these tissues diffuse action of the transmitter probably plays an important part in the normal transmission of impulses (Rosenblueth & Rioch, 1933), so that the situation would be somewhat comparable to that of diffuse application of ACh to denervated muscle.

The cause of supersensitivity. One of the immediate consequences of motor nerve section is an almost complete cessation of contraction in the muscles affected. It is therefore conceivable that supersensitivity may be due to inactivity of the muscles. This view seems to be supported by experiments of Solandt, Partridge & Hunter (1943) who found an increased sensitivity to intra-arterial injections of ACh in rat gastrocnemius-soleus muscles chronically immobilized by fixation of the knee and ankle joints.

Experiments on incompletely denervated muscle provide a crucial test for the hypothesis that lack of mechanical activity is an important factor in denervation supersensitivity. Most sartorius fibres have at least two end-plates, and section of the nerve to one of them does not prevent the transmission of impulses through the remaining junctions (visible reflex contractions of partially denervated sartorius were not abnormal). It follows then that if inactivity were the cause, sensitization should not develop in partially denervated fibres. Since this prediction is not correct,

sensitization must be related to a more direct influence of nerve on muscle, and not via changes in muscular activity. It is the removal of such an effect which causes supersensitivity.

As a consequence of nerve section the amount of ACh which impinges on an end-plate is considerably reduced. However, this is probably not causally connected with the increased ACh sensitivity since the latter was not abolished by prolonged application of ACh. The present situation differs from that described by Emmelin & Nordenfelt (1959), who found a long-lasting decrease in the supersensitivity of denervated cat's tongue muscle after repeated injections of ACh (cf. also Simeone, 1938*b*).

Thus one has to look for an action of the nerve which does not involve ACh. This influence could be exerted separately in time or it might be due to some substance liberated together with ACh, spontaneously during the production of m.e.p.p.s, or after arrival of nerve impulses.

Whatever the nature of this influence, it certainly controls the number and spatial spread of receptor units in the muscle membrane. Removal of the 'neural factor', as in denervation, causes them to proliferate along the fibre surface while its reappearance after reinnervation obliterates the 'extra-junctional' receptors (Miledi, unpublished). A puzzling problem is that the neural influence inhibits the spread of receptors beyond the synaptic region, but evidently fails to obliterate those in the synapse itself. The situation might be summarized tentatively by suggesting that the receptor molecules of the muscle fibre are being produced continually at the end-plate, from which they would 'overflow' and spread along the fibre surface but for the inhibiting influence of the motor nerve.

SUMMARY

1. Frog sartorius muscle fibres were examined after complete or partial denervation.

2. The area of membrane sensitive to acetylcholine increases after denervation, and in completely denervated fibres, some 10 weeks after nerve section, involves the whole fibre surface. However, even 158 days after complete denervation the maximum sensitivity is still found at the original end-plate regions.

3. The local sensitivity of denervated end-plates to close range application of brief iontophoretic pulses of acetylcholine was not significantly different from normal.

4. Desensitization during repetitive application of ACh pulses occurs in denervated muscle and is greatest at those points where sensitivity is relatively low.

5. Acetylcholine continuously applied for 5 days to denervated, isolated muscles (kept at 6° C) did not abolish their supersensitivity. At room

temperature, acetylcholine applied for 24 h did not abolish subsequent responses to ACh micro-application at the sensitized pelvic end.

6. Membrane potentials were recorded simultaneously from a denervated and an innervated end-plate in the same muscle fibre with intracellular electrodes. Miniature end-plate potentials at denervated end-plates remained normal during the first few days after nerve section; they then disappeared only to reappear, at a much lower frequency, a few days later. Miniature potentials at innervated end-plates were normal in frequency and amplitude.

7. There was no significant difference in the mean amplitude of miniature potentials between denervated and innervated end-plates in the same fibres.

8. The membrane resistance of partially denervated muscle fibres was greatest at the denervated end-plate region.

9. Denervated end-plates were more sensitive to *diffusely* applied acetylcholine than innervated end-plates in the same fibres, or in the control muscle.

10. Spontaneous miniature potentials, end-plate potentials, or potentials evoked by iontophoretically applied acetylcholine at *innervated* end-plates of supersensitive muscles were not significantly different from normal.

11. It is concluded that the motor nerve exerts a direct restraining action on the chemo-sensitivity of muscle. Such an influence is probably not mediated by acetylcholine. After denervation, it is not the muscular inactivity, but the removal of this neural influence which causes supersensitivity. The latter is brought about by an increase in chemo-sensitive membrane area with no actual increase in the sensitivity of the 'receptors' themselves.

I wish to express my gratitude to Professor B. Katz for constant help and encouragement. It was particularly helpful to discuss the work with him.

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JUNCTIONAL AND EXTRA-JUNCTIONAL ACETYLCHOLINE RECEPTORS IN SKELETAL MUSCLE FIBRES

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While examining the sensitivity of frog muscle fibres to acetylcholine (ACh) applied from a micro-electrode it was noticed that the reactive portion of the fibre appeared to be greater than the neuromuscular junction itself (Miledi, 1959). On account of the length and variability of the junctional nerve branches in the frog (Kühne, 1887; Couteaux, 1947; Cole, 1955), it is difficult to make there a proper comparison between the extent of the muscle fibre sensitive to ACh and that of the synaptic contact. It was therefore decided to repeat these experiments on neuromuscular junctions of the rat diaphragm, where the synaptic region occupies a circular surface area of only 19–28 μ diameter (Cole, 1957).

METHODS

Adult (300–350 g) male and female albino rats were used. The diaphragm was removed and the left posterior quarter-diaphragm mounted for intracellular recording (see Krnjević & Miledi, 1958*a*). ACh was applied by iontophoresis through a micro-electrode. In order to determine the ACh-sensitive portion of the muscle fibres a micro-electrode was inserted in a region where miniature end-plate potentials (m.e.p.p.s) could be recorded, and ACh was applied at different points of the fibre surface in steps of about 50–100 μ. The limits of the ACh-sensitive portion were taken as the last points at which a detectable ACh potential arose. The test doses of ACh used at these points were generally those released by approximately $1–2 \times 10^{-8}$ coulombs, delivered usually in 0·37 sec. Checks were repeatedly made that both micro-electrodes were in the same fibre by recording the electrotonic potential after insertion of the ACh pipette. When comparing local sensitivities these were expressed as the ratio between ACh potential (mV) and quantity of current (nC) which released the ACh (cf. Miledi, 1959).

RESULTS

The length of muscle fibre which responds to iontophoretically applied ACh varies from fibre to fibre. The mean ($\pm$ S.E.) for 18 fibres in four different muscles was $520 \pm 28\,\mu$: the shortest ACh-sensitive length encountered was 330 μ while the longest was 800 μ. Axelsson & Thesleff (1959) obtained responses to ACh only over not more than 100 μ of the fibre length; but this was probably due to the fact that the test doses of

ACh were smaller than those used in the present experiments. The distribution of ACh sensitivity along the reactive portion is such that there is a maximum which corresponds with the region where focal m.e.p.p.s, i.e. potentials rising to a peak in 1–2 msec, are recorded. From this point the local sensitivity falls more or less symmetrically on both sides and may be several thousand times smaller 100–200 μ away. This peaked spatial distribution of sensitivity resembles that of normal frog muscle fibres (Miledi, 1959).

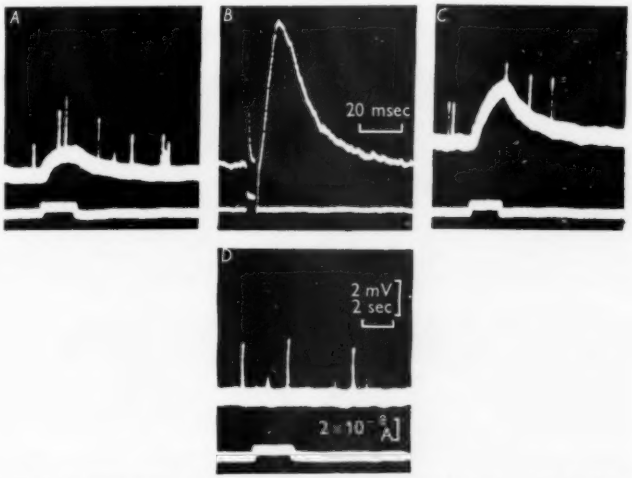

Fig. 1. Intracellular records of potentials evoked by microapplication of acetylcholine (ACh) to a muscle fibre in a rat's diaphragm. Spontaneous miniature endplate potentials appear as vertical lines in the upper traces. The current flowing through the ACh pipette is monitored in the lower traces.

A. Depolarization produced by ACh applied to the fibre at -320μ from the recording electrode. *B* and *C*. Same but ACh pipette at $+40$ and $+320\mu$, respectively, from recording electrode. *D*. No response to ACh applied at same longitudinal position as *B* but 90μ transversely away from the fibre. Time calibration for *A* and *C* as in *D*. Voltage and monitor trace calibration same for all records.

For example, in Fig. 1*B* a short pulse of ACh applied to the fibre 40μ away from the recording electrode produced a relatively brief depolarization. If the ACh was applied at a distance of 320μ at either side of the recording electrode a much longer pulse of ACh was needed to obtain a response, which was slower and smaller (Fig. 1*A* and *C*). Several tests, such as inverting the polarity of the pulse or inserting the micropipette, showed that these responses were ACh potentials and not electrotonic potentials caused by accidental insertion of the ACh pipette. No responses were obtained after moving the pipette a further 50–75 μ away: the total

sensitive length was therefore taken as 640 μ. Actually the sensitive length was probably slightly larger than this, because at a distance of about 300 μ the amplitude of the responses would be attenuated by electrotonic decrement to about one half.

The responses illustrated in Fig. 1 *A* and *C* cannot be due to ACh diffusing to the more sensitive region near the recording electrode, for no response was seen when similar pulses were applied at the same distance from the recording electrode as in *B* but 90 μ away from the edge of the fibre (Fig. 1 *D*). In fact, the ACh pipette can be moved at this distance parallel to the long axis of the fibre without eliciting a response in it. Furthermore,

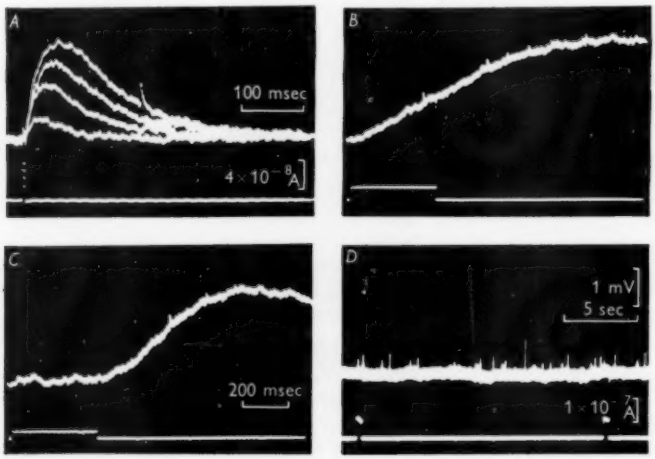

Fig. 2. Intracellular potential changes caused by ACh applied to a fibre at distances of 75 μ (*A*) and 375 μ (*B*) from the recording electrode. In *C* and *D* the ACh pipette was in the same longitudinal position as in *A* but 35 μ (*C*) or 100 μ (*D*) displaced transversely from the edge of the fibre. The pulse duration in *D* is the same as in *B* or *C*. Time calibration for *B* as in *C*. Voltage calibration same for all records. Monitor trace calibration for *B* and *C* as in *D*.

the relatively short latency of the ACh potentials also indicates that responses to ACh applied near the end of the sensitive portion are not due simply to diffusion of the substance to the synaptic region. It can be seen that a short pulse of ACh applied near the synaptic region produces a depolarization which starts after a very brief delay (Fig. 2 *A*). Again, if the ACh is applied 300 μ away along the fibre the depolarization begins soon after the start of the pulse and goes on increasing for some time after its end (Fig. 2 *B*). In contrast, if the ACh is applied at the same longitudinal position as in Fig. 2 *A*, but displaced about 35 μ tranversely from the edge of the fibre, the depolarization starts only after an appreciable

latency (Fig. 2*C*) while the general time course of the ensuing response is faster than that in Fig 2*B*. Here again, if the ACh pipette is moved transversely 100 μ away, no response is obtained even after large doses (Fig. 2*D*).

The evidence presented above strongly suggested that the responses to ACh applied at some distance from the neuromuscular junction were not due to diffusion of the drug to the more sensitive synaptic region. But it might be possible that the spread of the ACh towards the end-plate was considerably faster and more effective when applied directly to the fibre surface. It was therefore necessary to determine the site of origin of the

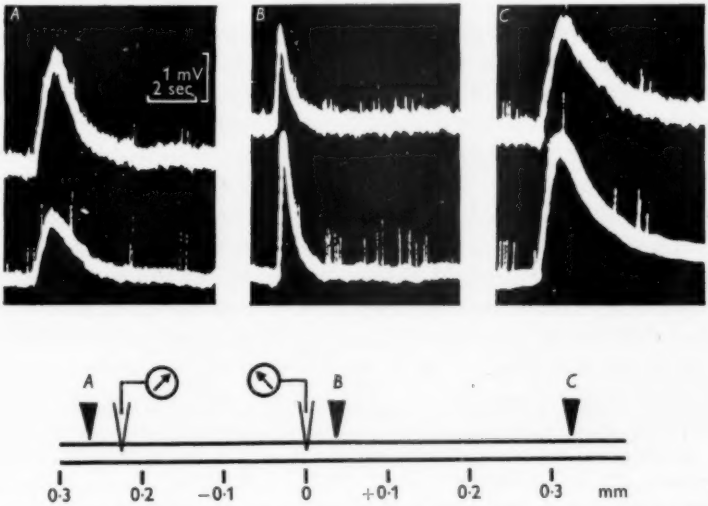

Fig. 3. Stimultaneous intracellular recording of potentials at two places in the same fibre. One micro-electrode (bottom traces) was near the neuromuscular junction; the other (top traces) was 225 μ away from the first. *A*, *B* and *C* illustrate potentials produced by a 0·37 sec pulse of ACh applied at -263μ, $+75\mu$ and $+325\mu$ respectively from the electrode placed near the junction (see diagram, where the positions of the ACh pipette are represented by black triangles). Voltage and time calibration for all records as in *A*. The intensity of ACh pulses was not monitored in this case but the ACh sensitivity at *B* was greater than at either *A* or *C*.

ACh potentials in each case. This was done by placing one micro-electrode in a region of focal m.e.p.p.s. A second micro-electrode was then inserted into the same fibre about 250 μ away from the first, and ACh was applied with an external pipette at different spots along the fibre. It is clear that if the ACh potentials arise from diffusion to the neuromuscular junction they should always appear larger at the focal micro-electrode.

In Fig. 3 the lower traces were recorded with a micro-electrode near the junction, and the top ones with an electrode placed 225 μ away. Miniature e.p.p.s are seen to appear simultaneously in both traces. In every single instance the amplitude of the spontaneous potentials was larger in the lower records from the focal micro-electrode (average amplitude 0·80 mV compared with 0·37 mV in the upper records), the difference being due to electrotonic decrement. When ACh was applied at A, i.e. a point $-263\,\mu$ from the focal electrode and 38 μ from the distant one, the amplitude of the ACh potential was larger in the latter and it spread electrotonically to the focal electrode (Fig. 3A). On the contrary, if the ACh was released at B or C, $+75$ or $+325\,\mu$ from the focal electrode (see diagram in Fig. 3), the potentials were then larger at this place. By applying the ACh between the two recording electrodes it was possible to equalize the depolarizations recorded at both places. Evidence of this type indicates that the responses originate quite close to the site of microapplication of ACh.

DISCUSSION

The extensive studies of Cole (1947, 1957) show that there is no great variation in the size of end-plates in adult rats. He finds (Cole, 1947) that after staining with gold chloride the width of the end-plate in the intercostal muscles was $29\,\mu \pm 2{\cdot}4$ (S.D.). From measurements of 3500 endings Cole (1957) mentions that in the rat diaphragm the diameter of the end-plate is 19–28 μ.

A disturbing feature could be the presence of double nerve endings, but these according to Cole (1955) were not very frequent. Furthermore, had they occurred they would probably have been detected either by the different time course and amplitude of their m.e.p.p.s or by the presence of an extra peak in the spatial distribution of ACh sensitivity; or else the two endings were so close together that the actual size of the synaptic regions was only doubled.

We have seen above that the length of the chemo-sensitive membrane is about 520 μ. Thus the picture which emerges is that besides the 'junctional' receptors situated under the nerve endings in the region of the junctional folds (see Robertson, 1957; del Castillo & Katz, 1957), there are 'extra-junctional' receptors covering an appreciable membrane area around the neuromuscular synapse. Receptors at these two sites probably have the same properties. The lower sensitivity and slower time course of the potentials at increasing distances from the synapse can be explained by a progressive decrease in the density of the extra-junctional receptors (cf. Miledi, 1959).

At distances greater than some 200–400 μ from the junction the receptors are either absent or present in such small numbers that one is unable to obtain an ACh potential from these regions. Ochs & Mukherjee (1959)

claim that in frog muscle ACh has a generalized action everywhere along the fibres. Their evidence, however, was based on long-term bath application of large doses of ACh, and it may be that during such a procedure leakage of K leads to a spread of depolarization along the fibre. In frog fibres (as well as in the rat's diaphragm) responses to microapplication of ACh *can only* be obtained from a region restricted to and closely surrounding the neuromuscular junction (Miledi, 1959).

The extra-junctional receptors occupy a wide band of the muscle fibre. This of course makes it quite easy to obtain responses to large doses of ACh. In every instance, out of about 40 fibres where m.e.p.p.s were detected it was possible to obtain ACh potentials (testing with about 1×10^{-11} coulombs in 40 msec). Nevertheless, brief ACh potentials comparable to end-plate potentials (cf. Krnjević & Miledi, 1958*b*) were difficult to obtain and for these the position of the ACh pipette was very critical. Moreover, in some fibres these fast responses could not be elicited at all: one would surmise that they are only obtainable from the synaptic region where the concentration of receptors presumably reaches a sharp peak.

One question that may be raised is whether the extra-junctional receptors are activated by the amount of ACh liberated normally by nerve impulses, especially during repetitive excitation. Under certain abnormal conditions, activation of extra-junctional receptors becomes rather more likely. Thus after inhibition of cholinesterase some of the ACh liberated by a nerve impulse may be able to diffuse to the extra-junctional receptors without being hydrolysed on the way. Such an event was indeed suggested by Eccles, Katz & Kuffler (1942) to explain the slow potential wave that is recorded at end-plates of eserinized muscle. These authors further found that small doses of curare greatly diminished the 'slow wave'. This might also be explained by the presence of extra-junctional receptors, for since their density is smaller than that at the junction the juxta-synaptic area may be more easily inactivated by curare. Again, it is conceivable that drugs may have an easier access to the comparatively exposed extra-junctional receptors.

It has previously been shown (Miledi, 1959) that the motor nerve exerts a long-term restraining action on the chemo-sensitivity of the muscle membrane. It is clear, nevertheless, that this neural influence does not altogether prevent the spread of receptors to the extra-junctional region even in normally innervated muscle. The extent of this region is probably not fixed and may decrease or increase, depending on the intensity of action of the controlling neural factor.

SUMMARY

1. Potentials were recorded intracellularly from rat diaphragm fibres while acetylcholine was applied by iontophoresis through a micro-electrode.

2. The length of fibre over which acetylcholine produced a detectable depolarization was $520 \pm 28\,\mu$ (mean $\pm$ S.E. for 18 fibres). The maximum of local sensitivity to acetylcholine coincides with the site at which focal miniature end-plate potentials are recorded. The sensitivity falls off at either side of this region and a few hundred microns away may be several thousand times smaller.

3. The time course of the responses to acetylcholine becomes slower with increasing distances from the neuromuscular junction.

4. The evidence indicates that the chemo-sensitive properties of the muscle fibre membrane extend well beyond the neuromuscular junction and that the density of extra-junctional acetylcholine receptors decreases with distance from the synapse.

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THE FRACTIONATION AND PURIFICATION OF FACTOR I

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Factor I action was originally defined by Florey (1954) as the ability of an extract of brain to abolish the discharge of the slow-adapting sensory neurone of the crayfish stretch receptor organ. Since that time it has been shown that Factor I is able also to affect a number of other structures, often to cause inhibition, but occasionally to augment the response (Florey & McLennan, 1955*a*, *b*, 1959; Florey, 1956; Honour & McLennan, 1960).

Bazemore, Elliott & Florey (1957) identified in crude extracts of brain gamma-aminobutyric acid (GABA), and showed that this substance has a potent blocking action on the crayfish stretch receptor neurone. However, the present author (McLennan, 1957; Honour & McLennan, 1960) has demonstrated that GABA mimics very few of the other actions of Factor I. Other compounds with activities towards the crayfish approaching that of GABA are the lower members of the series of omega-guanidino aliphatic acids (Edwards & Kuffler, 1959; McLennan, 1959); and paper chromatographic evidence suggested that some of these compounds were present in the extracts containing Factor I (McLennan, 1959). Nevertheless, like GABA they appear not to possess the other actions associated with Factor I (Honour & McLennan, 1960).

It has been reported earlier that paper chromatographic separation results in the appearance of two fractions of Factor I (McLennan, 1959). Both these fractions are active not only on the crayfish, but on the other structures affected by the unfractionated extracts as well (Honour & McLennan, 1960). The present paper describes attempts to purify these fractions further, and to identify the active constituents involved. This last aim has not been achieved; nevertheless, considerable purification of the Factor I fractions has been accomplished, such that the specific activity of one towards the crayfish is greater than that of GABA or of any other pure compound so far tested.

METHODS

Assay of Factor I activity. The crayfish stretch receptor neurone preparation, described originally by Florey (1954), has been used throughout this work as a routine test object for Factor I. Although possessing a high sensitivity to Factor I, the crayfish is unspecific (see, for example, Honour & McLennan, 1960); nevertheless, the speed with which the large numbers of samples involved in this study could be assayed rendered it the only object of practical use.

Samples to be applied to the stretch receptor were dissolved in the salt solution described by van Harreveld (1936). This solution could be used directly to elute material from dried paper chromatograms or to take up the residues remaining after evaporation of the effluent solutions from columns. Only in the case of papers which had been developed in phenol was special treatment necessary. These were washed, first in benzene and then in *n*-hexane, followed by heating for 1 hr at 90° C, in order to rid them of phenol, before being eluted for assay.

Factor I activities are expressed in this paper in terms of 'crayfish units reference' (c.u.r.), following the notation of Elliott & Florey (1956). A reference standard solution of Factor I was prepared by boiling 100 g chopped ox brain in 100 ml. water, centrifuging, and preserving the supernatant frozen. With most crayfish this supernatant could be diluted a hundredfold and still produce a perceptible block of the stretch receptor neurone discharge: it was therefore assigned an activity of 100 c.u.r./ml., and all unknown solutions have been compared with this standard.

The purified materials finally obtained were tested also for their actions on the inferior mesenteric ganglion of the rabbit *in vitro*. The dissection of the ganglion has been described by Brown & Pascoe (1952), and the action on it of Factor I by Florey & McLennan (1955*a*) and Honour & McLennan (1960).

Paper chromatography. Two-dimensional paper chromatograms on Whatman No. 4 paper ($22\frac{1}{2} \times 18\frac{1}{4}$ in., 57×46 cm) were run in sealed cabinets, at temperatures between 22 and 24° C. They were developed in a number of different solvent systems, details of which will be found under Results. Strips of Whatman No. 1 paper (36×1 in., $91 \times 2{\cdot}5$ cm) were also used.

Cellulose columns. For performing larger scale fractionations of Factor I into the two active fractions found on paper chromatograms, columns packed with cellulose powder (60×2 cm diameter) have been used. These were developed with the top layer of an *n*-butanol–acetic acid–water (4:1:5 by vol.) solvent mixture, and effluent fractions of 10 ml. collected. Portions of 0·1 ml. were dried off and taken up in the crayfish solution for assay as described above, and the effluent fractions showing activity corresponding to fractions A and B of Factor I previously described were pooled and worked up together.

Ion exchange chromatography. The techniques described by Hirs, Moore & Stein (1952) have in the main been employed in this study, with only minor modifications. Columns (25×3 cm diameter) were poured, either of Dowex-1-X8 (200–400 mesh) in an acetate form, or of Dowex-50W-X4 (200–400 mesh) in an ammonium form, and developed with 0·2M ammonium acetate buffer, pH 5·5, or 0·2M ammonium formate, pH 3·5, at 25° C. Effluent fractions of 20 ml. were collected, and again portions of 0·1 ml. were dried off and the residues taken up in the crayfish solution for assay. The volatile ammonium acetate or formate buffers were removed from active fractions by the method of Hirs *et al.* (1952), leaving residues whose appearance will be described below. Where an active extract was applied to a second column after elution from a first, the buffer was removed before the second fractionation.

Brain. Ox brain was obtained within minutes of the death of the animals by exsanguination, and stored in plastic bags set in ice until worked up. The brain stem and cerebellum were removed, and the remainder was cut into pieces with a sharp knife and dropped into boiling water. Details of the subsequent procedure, which in its initial stages differs little from that described by Florey & McLennan (1955*b*), are given below.

RESULTS

A flow sheet summarizing the procedure followed for the initial steps in the separation of Factor I activity from brain is given in Table 1, and Table 2 lists the quantities of Factor I found by assay on the crayfish at various

TABLE 1. Flow sheet for the initial steps involved in the separation of Factor I from brain. Roman numerals indicate steps to which further reference is made in Table 2

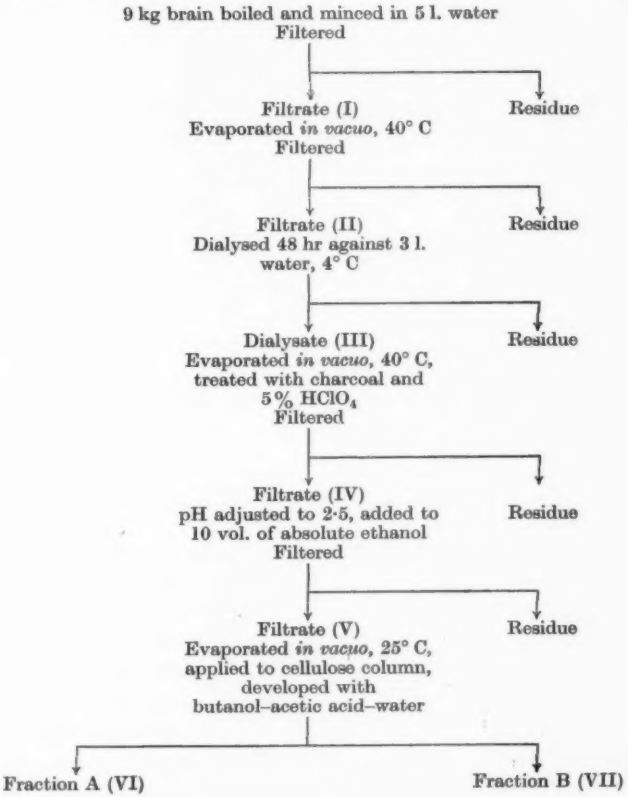

TABLE 2. Activities of fractions in initial Factor I separation: details in Table 1

Material	Total vol. (ml.)	Total solids (mg)	Total activity (c.u.r.)	Activity (c.u.r./mg)
Filtrate (I)	3570	66,500	142,800	2·1
Filtrate (II)	236	39,200	141,600	3·6
Dialysate (III)	3000	29,400	112,500	3·8
Filtrate (IV)	75	11,400	62,500	5·5
Filtrate (V)	750	5,600	60,000	11
Fraction A (VI)	—	130	40,000	310
Fraction B (VII)	—	200	16,800	84

stages in the course of the extraction. It is evident that the first few steps in this procedure do not result in much purification of Factor I, for the activity per milligram of dry material rises only by a factor of 3. The precipitation step with alcohol removes much inactive material, and the specific activity is doubled.

All the fractions collected from the cellulose column were assayed for their Factor I content, and it was evident that the original activity had separated into two bands. The appropriate fractions were pooled, and portions (containing *ca.* 10 c.u.r.) applied to filter-paper strips, developed with aqueous phenol (80 %), and sprayed with ninhydrin. Duplicate strips were cut up and eluted, and tested again on the crayfish. The R_F values obtained for the activity and for the ninhydrin spots are shown in Table 3.

TABLE 3. Chromatographic data on active Factor I fractions separated on cellulose columns

	Cellulose column (R)	R_F in aqueous phenol of		
		Factor I activity	Ninhydrin spots	Possible identification of ninhydrin spots
Fraction A	0·16 (4)*	0·28 (4)	0·23 (3)	Glutamic acid, Aspartic acid, Serine
			0·52 (4)	Glutamine, Alanine
			0·73 (4)	Lysine, Glucosamine
Fraction B	0·42 (4)	0·72 (4)	0·85 (1)	GABA

* Number of observations in parentheses.

As was to be expected, the two active bands eluted from the cellulose columns corresponded closely to the fractions, called A and B, which were identified by paper chromatography of Factor I solutions (McLennan, 1959). The data summarized in Table 3 were obtained with two batches of Factor I, each run through two columns. The table includes the possible identification of the ninhydrin spots as amino acids known to be present in the brain, having regard to the R_F values found with phenol and the R values from the columns. None of the compounds thus tentatively identified in the active fractions possess inhibitory activity towards the crayfish, except, of course, the GABA which may have been present in one sample of fraction B. Glutamic acid does have an occasional effect on the stretch receptor neurone, but this takes the form of excitation which may lead to a block of impulse discharge if the solution is allowed to remain in contact for a prolonged period. This effect has been noted also by Elliott & van Gelder (1958).

It seemed clear from these observations that the active material of fraction B almost certainly, and of fraction A probably, was not associated

with compounds capable of reaction with ninhydrin, at least within the limits of sensitivity of the method. Further dissociation of the Factor I activity and ninhydrin-reacting material could be achieved by the use of Dowex-50 W resin, and development with mixed citrate and acetate buffer, as described by Moore & Stein (1954). Again the effluent fractions were assayed for Factor I, and those showing activity applied to paper strips and developed with aqueous phenol. The active eluates from columns charged both with fractions A and B now showed no evidence of the presence of ninhydrin-reacting compounds.

TABLE 4. Flow sheet for the ion-exchange chromatography of Factor I fractions (see also Table 5)

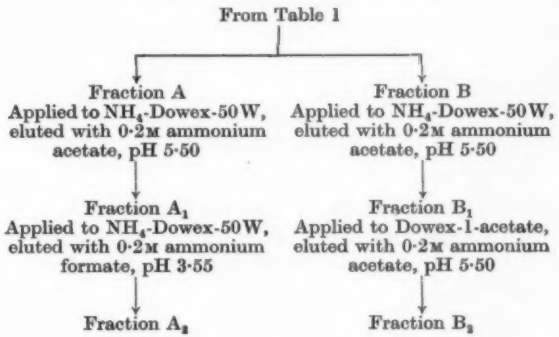
From Table 1
Fraction A
Applied to NH_4-Dowex-50W, eluted with 0·2M ammonium acetate, pH 5·50
Fraction B
Applied to NH_4-Dowex-50W, eluted with 0·2M ammonium acetate, pH 5·50
Fraction A_1
Applied to NH_4-Dowex-50W, eluted with 0·2M ammonium formate, pH 3·55
Fraction B_1
Applied to Dowex-1-acetate, eluted with 0·2M ammonium acetate, pH 5·50
Fraction A_2
Fraction B_2

TABLE 5. Activities of fractions in Factor I separation: details in Table 4

Material	Total solids (mg)	Total activity (c.u.r.)	Activity (c.u.r./mg)	Appearance of dried material
Fraction A_1	90	32,000	330	Small, irregular, greasy crystals
Fraction B_1	160	16,000	100	Oil
Fraction A_2	25	28,000	1120	Monoclinic crystals, very deliquescent
Fraction B_2	80	16,000	200	White, amorphous

In order to attempt the isolation of the active ingredients of the Factor I fractions, ion exchange chromatography was used, and the columns eluted with volatile buffer systems. The further progress of the purification is outlined in the flow sheet of Table 4, and additional data are given in Table 5. Fractions A and B were differently absorbed by the ion exchange resins used. Fraction B seems likely to be uncharged, since it passed through columns of both Dowex-50 W and Dowex-1 without hindrance. Fraction A was strongly absorbed by Dowex-50 W both at pH 5·5 and at pH 3·5, and must therefore possess a cationic group.

The two fractions obtained following ion exchange chromatography

possessed together 56,800 c.u.r. of Factor I, out of a total of 60,000 remaining from the original and before fractionation was begun (step V in Table 1). The specific activities of the solid material shows that a considerable purification has been achieved. Honour & McLennan (1960) have shown that less purified A and B fractions of Factor I cause a depression of the post-synaptic responses of the inferior mesenteric ganglion of the rabbit, and are roughly equally potent in this respect. Accordingly,

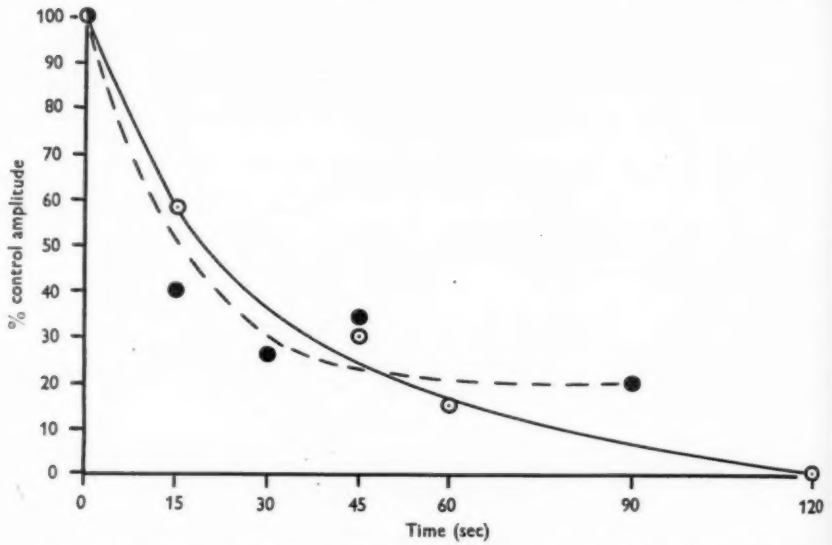

Fig. 1. Time courses of depression in amplitude of the post-synaptic response of the rabbit's inferior mesenteric ganglion, following application of purified Factor I fractions at zero time. ● — ●, 1 drop fraction A_2, 140 c.u.r./ml.; ⊙ — ⊙ 1 drop fraction B_2, 160 c.u.r./ml.

solutions of approximately equal concentrations of the purified fractions were similarly applied to the ganglion *in vitro*. The time courses of the depression in amplitude of the post-synaptic response are shown in Fig. 1, and their form is indistinguishable from those reported earlier for the less purified material.

Small portions (*ca.* 50 c.u.r.) of the active residues were dissolved in water and placed on papers, and subjected to two-dimensional development. The papers were then cut up and eluted in the usual way to determine the position of the activity. As noted above, none of the fractions gave evidence for the presence in them of material capable of reaction with ninhydrin. The chromatographic data for the two fractions are set forth in Table 6.

TABLE 6. Paper chromatographic data for the purified Factor I fractions, A_2 and B_2

Solvent system	R_F values for Fraction A_2	R_F values for Fraction B_2
n-Butanol–acetic acid–water (4:1:5 by vol.)	0·18 (4)*	0·43 (5)
n-Butanol, saturated with water	0·12 (2)	0·12 (2)
n-Propanol–acetic acid–water (73:10:17 by vol.)	0·42 (3)	0·54 (2)
Pyridine–isoamylol–acetic acid–water (8:4:1:4 by vol.)	0·27 (2)	0·40 (2)
Lutidine–collidine–water (1:1:1 by vol.)	0·15 (2)	0·18 (2)
Phenol–water (80:20)	0·43 (3)	0·83 (2)

* Number of determinations in parentheses.

DISCUSSION

This investigation was undertaken on the basis of several earlier reports: (*a*) that brain extracts contained a factor (Factor I) causing inhibition of discharge of the crayfish stretch receptor neurone (Florey, 1954); (*b*) that similar extracts had a variety of actions, mostly inhibitory, in mammalian nervous systems (Florey & McLennan, 1955*a*, *b*; Honour & McLennan, 1960); (*c*) that GABA, known to be present in the brain, was a potent inhibitor of the stretch receptor neurone (Bazemore *et al.* 1957) but did not mimic the mammalian actions of Factor I (McLennan, 1957; Honour & McLennan, 1960) and was not a necessary constituent of the brain extracts active on the crayfish (McLennan, 1958); and (*d*) that various guanidino acids, also active on the crayfish (Edwards & Kuffler, 1959) and probably present in the brain extracts (McLennan, 1959), are similarly inactive in mammalian systems (Honour & McLennan, 1960). It was evident then that the ingredient present in the extracts of brain and responsible for the actions on mammalian systems unaffected by GABA and the guanidino acids remained to be identified.

The two fractions of Factor I which have been purified considerably, were recognized earlier on paper chromatograms (McLennan, 1959), and both were shown to possess actions on mammalian structures exhibited by crude Factor I (Honour & McLennan, 1960). In spite of their chromatographic differences, their actions were remarkably similar, suggesting that they might be in some way related chemically. These characteristics have not been changed in the course of purification, for the qualitative and quantitative relationships for the actions of the crude A and B fractions on the rabbit's inferior mesenteric ganglion (Honour & McLennan, 1960) are retained for the most purified preparations. The chromatographic behaviour of the fractions on paper is likewise unaffected by the purification (compare Table 6 with Table 1 in McLennan (1959)).

Fraction A has been purified to the extent that its final activity was in excess of 1000 c.u.r./mg, and a crystalline product resulted. However, the

extremely deliquescent nature of these crystals has prevented their further study, and they have not been characterized. It would appear likely that fraction B was still contaminated with inactive material, since its final specific activity was lower (200 c.u.r./mg) and a crystalline product was not obtained. In this connexion it is worth noting that GABA and guanidino-acetic acid, hitherto the most active compounds tested on the crayfish (Edwards & Kuffler, 1959; McLennan, 1959) have specific activities in the range 300–500 c.u.r./mg (Bazemore *et al.* 1957; McLennan, 1957, 1959).

It should be repeated that fraction B was not absorbed either by an anionic or by a cationic resin, in contrast to A which reacted with a strongly acidic resin. The differing mobilities in the solvent systems used for paper chromatography are probably related to the fact that only one of the fractions appears to have a charged group. The finding that throughout the purification the ratio

$$\frac{\text{crayfish activity A}}{\text{crayfish activity B}} = \frac{\text{ganglion activity A}}{\text{ganglion activity B}} = \frac{1}{1}$$

supports the suggestion that the same active substance is involved in both fractions, but that in one case it is present as an uncharged complex. The final proof of this, however, with identification of the compounds involved, has not been achieved.

SUMMARY

1. Brain extracts containing an inhibitory principle (Factor I) have been subjected to various purifying and fractionating procedures. Two active fractions were obtained; the one of higher specific activity yielded deliquescent crystals, the other was not crystallized.

2. The paper chromatographic characteristics of the two fractions in a number of solvent systems are given. Neither fraction appears to possess a ninhydrin-sensitive group.

3. It is suggested that the two fractions owe their activity to the same compound, which in one case is held in an uncharged complex and in the other exists as a free cation.

4. The final identification of this postulated active compound has not been achieved.

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THE RESPONSE OF THE THYROID GLAND OF THE GUINEA-PIG TO STRESS

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Various procedures that are known to cause an increase in adrenocortical activity through a reflex discharge of adrenocorticotrophic hormone (ACTH) from the anterior pituitary have been shown to decrease the activity of the thyroid gland of rabbits and rats (Brown-Grant, Harris & Reichlin, 1954*a*, 1957; Brown-Grant, 1956*b*). A reciprocal balance between secretion of ACTH and thyrotrophic hormone (TSH) has been suggested as a general feature of the anterior pituitary response to stress (Harris, 1955). Recently Gerwing, Long & Pitt-Rivers (1958) have suggested that in other species (guinea-pig, monkey and man) stress may produce an increase in thyroid activity. The basis of this suggestion is their observation that bacterial exotoxins appear to increase the rate of release of radio-iodine from the thyroid glands of guinea-pigs and Rhesus monkeys. This striking species difference, together with the fact that these workers limited their studies to one type of stress stimulus, suggested that an investigation of the response to other types of stress should be carried out. The effect of various experimental procedures on the rate of release of thyroidal ^{131}I in the guinea-pig has been examined, and the results are presented here.

METHODS

Male guinea-pigs, 200–500 g body weight (b.wt.) of mixed strains were used in all experiments except one, which was performed on female guinea-pigs. The animals were kept in a temperature-controlled animal room (18–21° C) under constant conditions of lighting, and fed on Bruce & Parkes (1947) pelleted diet 18 plus tap water *ad lib.*, supplemented by a small amount of fresh cabbage five days a week.

Thyroid activity was determined as follows. Groups of three to six animals within a range of ± 30 g b.wt. were used in each experiment. They were injected with 20–40 μc of carrier-free radio-iodine (^{131}I, obtained from the Radio Chemical Centre, Amersham) intraperitoneally or intramuscularly. Radio-iodine in the thyroid was measured with a shielded gamma-ray sensitive Geiger–Muller tube (G 10 Pb, 20th Century Electronics Ltd.) operating a conventional automatic scaler through a quenching pre-amplifier. The rectangular window in the lead shield of the tube measured 3·5 cm in the long axis of the tube by 2·0 cm and the edges were shaped so that the conscious animals could be held on a raised

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wooden platform at right angles to the tube in a comfortable position without struggling and with the thyroid region in contact with the tube. The relatively large window was chosen to ensure a reasonable degree of reproducibility in counting rates despite minor inadvertent variations in the positioning of the animals. Five counts each of 100 sec duration were made by each of us on a series of twelve animals, which were removed and replaced between each count. The variation between counts rarely exceeded the range $\pm 5\%$ of the mean, which is about the expected statistical fluctuation expected at the counting rates employed. The sensitivity of the apparatus was determined by carrying out careful counts *in vivo* on a series of nineteen animals which were then killed, the thyroids dissolved in 3N-NaOH and the ^{131}I content determined by β-counting in an M6 liquid counter. It was found that 1 μc in the thyroid gland gave 8369 ± 181 counts/100 sec *in vivo* (S.E. of mean of nineteen determinations).

In the actual experiments the first thyroid counts were made 48–72 hr after injection of ^{131}I. Three 100 sec counts were averaged and the mean value, after subtraction of background, was taken as 100%. Initial counts were between 1000 and 3000/100 sec in most experiments. Each of the subsequent twice-daily counts, after correction for isotope decay, was expressed as a percentage of the initial value, and the logarithm of the mean percentage content for the group as a whole was plotted against time to obtain the release curve. This technique for measuring the rate of release of thyroidal ^{131}I was checked in various ways. The contribution of non-thyroidal radio-iodine to the 'thyroid count' was estimated by taking several animals 48–72 hr after injection of ^{131}I and making neck counts before and after removing the thyroid; the counting rate minus the thyroid was $3{\cdot}5 \pm 0{\cdot}7\%$ (S.E. of the mean of seven determinations) of the previous rate. Further, the release curves plotted on a semi-log. scale, as described above, were linear from the start and for periods up to 16 days in preliminary experiments. The uptake of ^{131}I by the guinea-pig thyroid was very low in these experiments. From the counts *in vivo* at 48 hr after injection a mean value of $1{\cdot}5 \pm 0{\cdot}2\%$ of the injected dose (S.E. of mean of thirty-three animals) was calculated on the basis of the known sensitivity of the Geiger counter. The reason for this low uptake is not known. The pellets which the animals ate *ad lib.* are reported by the manufacturers to contain only about 0·00046% by weight of iodine. The restricted supplement of cabbage did not contain any significant amount of goitrogen, as withdrawal of this and the substitution of ascorbic acid in the drinking water had no effect on the uptake. Further, rabbits in the same animal room fed on the same pellet diet and the same cabbage had 48 hr thyroid uptakes of between 18 and 25% of the administered dose, which is in the range observed in previous studies in another laboratory (Brown-Grant *et al.* 1957). Guinea-pigs eating pellets as their major source of food drink large quantities of water and the daily urine output of our animals was between 100 and 150 ml. Urinary excretion of iodide was rapid, as judged by the low blood levels and tissue background found 24–48 hr after injection, and it appeared that this might be in part responsible for the low thyroid uptake. However, when water was withheld for the first 12 hr after injection of ^{131}I, or for 8 hr before injection, only a slight (20%) increase in the 24 hr uptake was observed. Histological and autoradiographic examination of the thyroids of animals from the laboratory colony showed that the major portion of the gland was composed of large inactive follicles and we have concluded that this low uptake represents an extreme example of the well known tendency towards a reduced level of thyroid gland function in this species.

One consequence of this low uptake, however, is that in the interpretation of acute changes in the slope of the release curve, recirculation of iodine from degraded hormone can be neglected. Even if de-iodination were the sole route of metabolism of thyroid hormone in this species, which is exceedingly unlikely to be the case, recirculation would normally be only 1·5%. Under the conditions of the experiments described later, it is conceivable that the uptake could be increased at a time when the rate of release is decreased but even a three or fourfold increase would not account for the changes in the slope that

were observed. All the experiments reported were therefore carried out without the use of thiouracil or similar drugs to prevent recirculation. In preliminary experiments replacing the drinking water by a saturated solution of 4-methyl-2-thiouracil had no detectable effect on the rate of release for periods up to 7 days, as would be expected in the absence of significant recirculation. Animals treated in this way were receiving an adequate amount of the drug, as was shown by their failure to accumulate ^{131}I in the thyroid after injection as compared with a control group, and by histological evidence of thyroid activation after several weeks' treatment.

A further check on the validity of the release curve technique was made as follows: a group of five animals (260 g mean b.wt.) were injected with a suspension of 50 μg of L-thyroxine (Eltroxin, Glaxo Laboratories Ltd) intraperitoneally during a release curve. There was complete inhibition of ^{131}I release for a period of 36 hr, followed by a return to the previous slope (Fig. 1). A second group of five animals (380 g mean b.wt.) received 100 μg; the characteristics of the response were the same in this case, but the duration of inhibition was 68 hr.

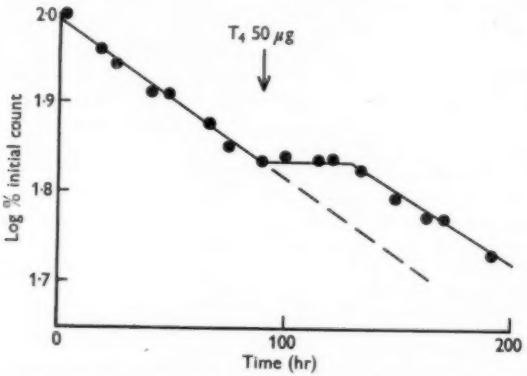

Fig. 1. The effect of 50 μg of L-thyroxine on the release of ^{131}I from the guinea-pig thyroid.

Various drugs and hormones were used in these experiments. Details of dosage and route of administration and the descriptions of the stress stimuli employed are given under Results. The effects of saline injections alone were checked in the following experiment. The rate of release of ^{131}I was measured for 4 days in a group of six animals (350 g mean b.wt.). On successive days each animal received 1 ml. of 0·9% (w/v) NaCl solution intraperitoneally, 1 ml. intramuscularly, 1 ml. subcutaneously and finally in the morning and in the evening 3 ml. of 4%(w/v) NaCl solution intraperitoneally. No effect on the rate of release was observed.

RESULTS

The response of the thyroid gland to stress

Noise. A group of five animals (260 g mean b.wt.) was exposed to the noise produced by banging various metal cans and glass beakers together for 10–15 sec, which was recorded on a tape recorder. A continuous loop of tape was prepared so that the noise was repeated every 30–40 sec. The maximum output of the tape recorder was fed to an audio-frequency

amplifier and played through an 8 in. (20 cm) speaker placed on top of the animal cage. The recording tape began to wear out after 8–10 hr continuous playing and was replaced at these intervals. The noise produced was unpleasant but not painful to the human ear placed close to the speaker. The animals were exposed to this noise continuously for 60 hr except for two periods in each day of about 1 hr when neck counts were made or when the recording tape was being replaced. The effect on the release curve was to produce a prompt and complete inhibition of ^{131}I release, followed by a return to the previous rate of release when the noise stopped (Fig. 2). The animals ate normally throughout the experiment and did not lose weight.

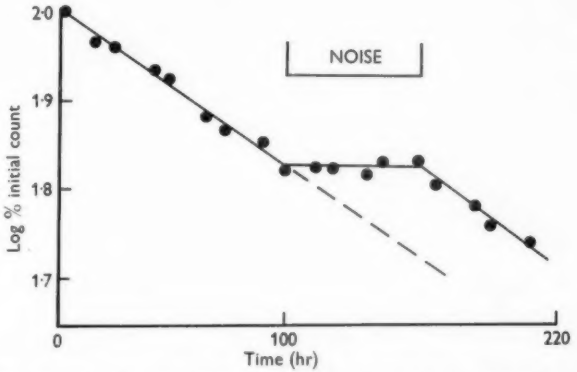

Fig. 2. The effect of loud noise on the rate of release of ^{131}I from the guinea-pig thyroid.

Cold. A group of three animals (420 g mean b.wt.) showed an increase in release rate following exposure for 70 hr to a temperature of $11 \pm 1°$ C (Fig. 3). Acute exposure to low temperature ($< 5°$ C) is known to be a stimulus to ACTH release and has been shown to produce a decrease in the thyroid activity of rabbits and rats (Brown-Grant, von Euler, Harris & Reichlin, 1954; Brown-Grant, 1956*a*). A group of three guinea-pigs (380 g mean b.wt.) exposed for 80 hr at $3 \pm 1°$ C showed a decrease in the rate of release of thyroidal ^{131}I (Fig. 4).

Surgical trauma. A group of three animals (285 g mean b.wt.) was subjected to laparotomy under ether anaesthesia during a release curve. Loops of small intestine were exposed through a 2 in. (5 cm) mid-line abdominal incision. After 20 min the intestines were returned to the abdominal cavity and the muscle and skin layers closed separately with continuous linen sutures. This procedure had no effect on the rate of release of ^{131}I from the thyroid (Fig. 5). It is of interest that surgical procedures of short duration also fail to produce significant ACTH release

in the guinea-pig as judged by urinary excretion of adrenal corticoids (Sobel, Schapiro & Marmorston, 1958).

Skin damage. Four female guinea-pigs (320 g mean b.wt.) were injected with 0·2 ml. of turpentine intradermally in the mid line of the back over the lumbar vertebrae. The initial irritation and erythema were followed

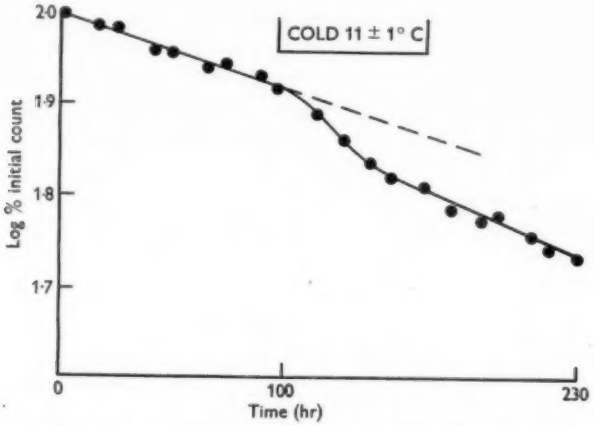

Fig. 3. The effect of exposure to moderate cold on the release of ^{131}I from the guinea-pig thyroid

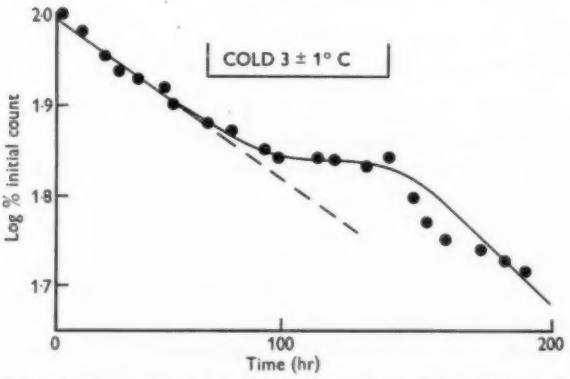

Fig. 4. The effect of exposure to severe cold on the rate of release of ^{131}I from the guinea-pig thyroid.

by the development of indolent ulcers. The release of ^{131}I stopped abruptly at the time of injection and the inhibition remained complete for 70 hr after the intradermal injection, after which there was a return to the previous rate of release.

Adrenaline. The adrenaline salt of mucic acid ('Hyperduric Adrenaline', Allen and Hanburys) was injected subcutaneously at doses of 100 μg twice daily for 2 days and 150 μg twice daily for a third day (total dose, 700 μg/animal). A group of five animals (410 g mean b.wt.) showed a prompt and complete inhibition lasting for 70 hr, followed by a return to the previous slope (Fig. 6).

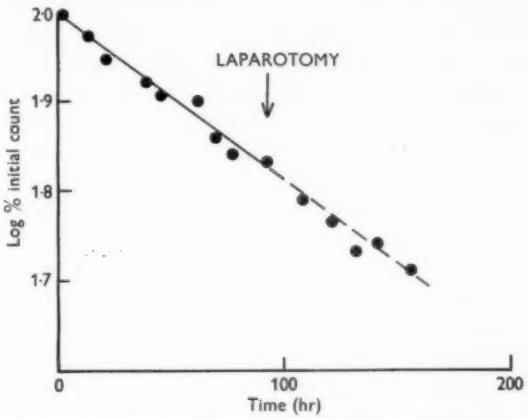

Fig. 5. Lack of effect of surgical trauma on ^{131}I release.

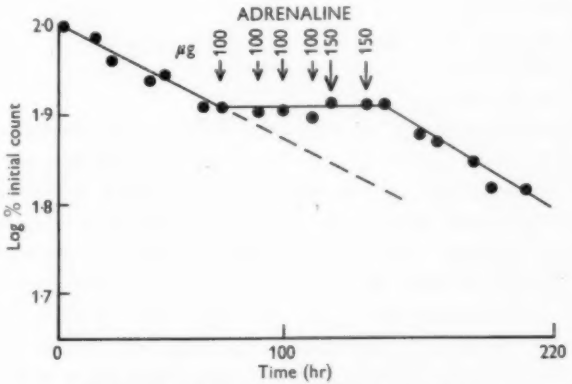

Fig. 6. The effect of adrenaline on ^{131}I release.

Vasopressin. Pitressin (Parke, Davis and Co.) has been shown to produce an increased secretion of urinary corticoids in the guinea-pig (Sobel, Levy, Marmorston, Schapiro & Rosenfeld, 1955). In the same dosage (5 u. twice daily intraperitoneally in 3·0 ml. of 4·0 % (w/v) NaCl solution, total dose 50 u./animal) a group of four animals (315 g mean b.wt.)

showed a prompt and marked decrease in ^{131}I release during the 120 hr period of administration, followed by a return to the previous rate of release. The result was similar to that obtained with adrenaline (Fig. 6), but the inhibition was not complete; a steady release rate of 1 %/day was observed during the period of the injections.

Typhoid vaccine. The dose used was 0·5 ml. of T.A.B. vaccine (Burroughs Wellcome and Co.) containing 500×10^6 *Salmonella typhi*, 250×10^6 *S. paratyphi* A and 250×10^6 *S. paratyphi* B organisms and was administered intramuscularly. A group of five animals (280 g mean b.wt.) showed a prompt and complete inhibition lasting 80 hr following a single injection of vaccine, similar to the responses illustrated in Figs. 1 and 6.

Corynebacterium diphtheriae exotoxin. Through the courtesy of Dr C. G. Pope of the Wellcome Research Laboratories, we were able to obtain a highly purified *Corynebacterium diphtheriae* exotoxin (Batch No. TP 3116) similar to that used by Gerwing *et al.* (1958) in their experiments. In the first experiment a group of five animals (240 g mean b.wt.) each received approximately 30 international Shick units of toxin intramuscularly and the same dose was repeated 24 hr later. The effect was prompt and complete inhibition of ^{131}I release, which persisted until the experiment was terminated by the death of four out of the five animals 60–80 hr after the first injection of toxin. At autopsy the typical haemorrhagic changes in the adrenal cortex were observed. In a second experiment, on a group of five animals (270 g mean b.wt.) that received a single injection of 30 i.u. of toxin, a complete inhibition of release for 40 hr followed by a return to the previous slope was observed. The response was indistinguishable from those illustrated in Figs. 1 and 6.

Electroconvulsions. It has been reported that electrically induced seizures result in an increased thyroid activity in the guinea-pig, as judged by histological examination of the thyroid gland (Ellis & Wiersma, 1945; Del Conte, Ravello & Stux, 1955). However, there is also clinical and experimental evidence that an increase in adrenal activity may be produced (Rosvold, Kaplan & Stevens, 1952; Reiss, 1954), in which case a decrease in thyroid activity might be expected. In one experiment a group of three animals (470 g mean b.wt.) were anaesthetized with ether and subjected to an electrically induced convulsion once daily for 3 days during a release curve. (The electrodes were an occipital skin electrode and one placed against the base of the skull within the mouth; stimulation parameters, 50 c/s a.c., 50 V, 90–100 mA, duration 30 sec.) The results were not clear-cut; no increase in the rate of release of ^{131}I from the thyroid was observed—possibly there was a slight decrease. A second experiment in which a group of six animals (330 g mean b.wt.) were subjected to similar daily shocks of 20 sec duration for 3 days was carried out. The

result in this case was a prompt and marked decrease in the rate of release to less than 1 %/day for 72 hr, followed by a return to the previous rate of release. The response was very similar to that seen after adrenaline or Pitressin administration (Fig. 6).

The response of the thyroid gland to ACTH and hydrocortisone

The guinea-pig has been described as a 'cortisone resistant' species on the basis of certain responses of immunological importance (Long, 1957); Gerwing *et al.* (1958) have suggested that their failure to produce a decrease in thyroid activity by the administration of bacterial exotoxins in this species may be related to this. The decrease in thyroid activity produced

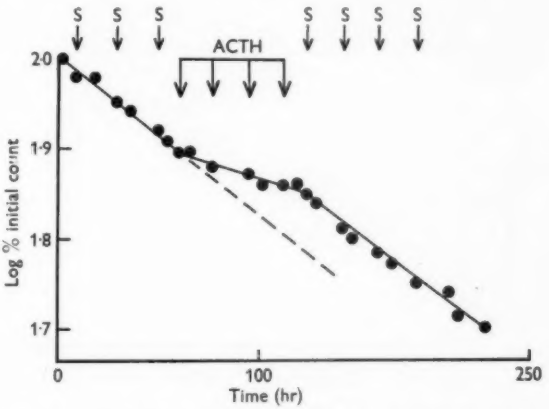

Fig. 7. Effect of ACTH (8 i.u. daily for 4 days) on ^{131}I release. S indicates control injection of 0·2 ml. saline intraperitoneally.

by large doses of ACTH or glucocorticoids in rats, rabbits and man is well established (Brown-Grant, Harris & Reichlin, 1954*b*); the response of the guinea-pig thyroid to ACTH and hydrocortisone (Compound F, the major glucocorticoid secreted by the guinea-pig adrenal) was examined.

ACTH. The preparation used was Crookes 'Cortico-gel' and the dose was 8 i.u. (0·2 ml.) intraperitoneally daily (total dose 32 i.u./g-pig). Control injections of 0·2 ml. 0·9 % (w/v) NaCl solution were given before and after the ACTH. The result, in a group of four animals (480 g mean b.wt.), was a marked decrease in the rate of ^{131}I release (Fig. 7).

Hydrocortisone. A dose of 2·5 mg twice daily intramuscularly (total dose 20 mg/g-pig) hydrocortisone acetate (Roussel Laboratories Ltd) produced a complete inhibition of ^{131}I release during the period of injection, followed by a return to the previous slope, in a group of five animals (250 g mean

b.wt.) (compare Figs. 1 and 6). Control injections of 0·1 ml. saline intramuscularly given before and after the hydrocortisone injections had no effect on the rate of release.

DISCUSSION

The release curve obtained by repeated measurements *in vivo* of the radio-iodine content of the thyroid gland represents the release of ^{131}I-labelled hormones, and provided that certain precautions have been taken, as has been discussed under Methods, provides an almost continuous record of the level of thyroid activity. The slope of the release curve *per se* gives no direct indication of the absolute level of thyroid activity, but by the use of an initial and final control period before and after exposure to the experimental stimulus under investigation valid deductions as to changes in thyroid activity and, tentatively, in TSH secretion may be drawn from changes in the rate of release (Brown-Grant, von Euler *et al.* 1954). Although a statistical evaluation of the data is simpler if the release rates of a control and an experimental group of animals are compared, the experimental design which allows the animals to serve as their own controls seems preferable when the effect of a given stimulus on thyroid activity is under investigation.

The effect of a variety of potentially harmful or disturbing procedures on the thyroid activity of the guinea-pig has been investigated in a series of experiments of this type. These procedures have little in common except that it is known, or highly probable, that they cause a reflex discharge of ACTH from the anterior pituitary. Collectively they may be referred to as 'stresses' and the results of these experiments indicate that their application results in a decrease in the thyroid activity of the guinea-pig, as had previously been found to be the case for rabbits and rats (Brown-Grant, Harris *et al.* 1954*a*; Brown-Grant, 1956*b*). Where a direct comparison can be made between these results and those of Gerwing *et al.* (1958) (administration of 30 i.u. of diphtheria toxin) their finding of an increase in thyroid activity could not be confirmed. It should be pointed out, however, that many of their experiments and also those reported by Gerwing (1958) were of much longer duration than any that we have carried out. In the lengthy experiments factors such as variation in food intake may alter the total (stable) iodide content of the thyroid; under such circumstances variations in ^{131}I release rate are no longer a valid index of the level of thyroid activity (Brown-Grant, von Euler *et al.* 1954). A second possibility exists to account for the difference between our results and those of Gerwing *et al.* The secretion of TSH can be imagined as being subject to a number of influences, some excitatory and others inhibitory. The eventual level of TSH secretion is determined by the

algebraic sum of these stimuli, somewhat in the way that the activity of a motor neurone of the anterior horn is determined. It is possible that in the experiments of Gerwing *et al.* the non-specific inhibitory stress effect of the toxins administered was outweighed by some other excitatory stimulus, perhaps a change in body temperature or the rate of metabolism of thyroxine resulting from the action of the toxins, so that in their experiments the over-all effect was excitatory. Such a concept of a balance between the various factors affecting TSH secretion has some experimental support. Acute exposure to progressively more severe cold may produce an increase, no change and finally a decrease in thyroid activity (Brown-Grant, 1956*a*). Reichlin & Lieberman (1958) have shown that the stress inhibition of the rat thyroid resulting from the surgical removal of 10–20 % of the skin may be preceded by a phase of thyroid over-activity, possibly due to the increased heat loss involved, whereas other surgical procedures such as sham adrenalectomy produce only an immediate decrease in thyroid activity in the rat (Brown-Grant, 1956*b*).

The decrease in thyroid activity produced by stress is not the direct result of an increased production of adrenocortical hormones, as it occurs in the adrenalectomized rabbit and rat (Brown-Grant, Harris *et al.* 1954*a*; Brown-Grant, 1956*b*; Reichlin & Glaser, 1958). Thus the suggestion of Gerwing *et al.* (1958), that the anomalous response observed in their experiments was due to an insensitivity of the guinea-pig thyroid to glucocorticoids, appears unlikely to be the explanation for their findings. Further, although in some respects the guinea-pig may be a 'cortisone resistant' species (Long, 1957), it shows, like the rat, rabbit and man, a decrease in thyroid activity under the influence of large doses of ACTH or hydrocortisone.

SUMMARY

1. A technique for obtaining thyroid ^{131}I release curves in the conscious guinea-pig is described and evaluated.

2. The responses to various stress stimuli (noise, cold, tissue damage, adrenaline, Pitressin, typhoid vaccine, diphtheria toxin, electroconvulsions) were investigated.

3. In each case a decrease in the rate of release of ^{131}I from the thyroid was observed.

4. The administration of large doses of ACTH or hydrocortisone also reduces the rate of release of ^{131}I.

5. It is concluded that the guinea-pig responds to acute stress with a prompt but reversible decrease in thyroid activity, as has been observed in other species.

6. The relationship of these findings to earlier reports that certain forms of stress increase the thyroid activity of the guinea-pig is discussed.

This work was carried out during the tenure of one of us (G.P.) of an International Atomic Energy Agency Fellowship, which is gratefully acknowledged, as is the hospitality extended by the head of the department, Professor H. P. Gilding. We would like to thank Mr M. Sherwood for his care of the animals and his assistance with these experiments.

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THE ACTIVE TRANSFER OF D-METHIONINE BY THE RAT INTESTINE *IN VITRO*

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Gibson & Wiseman (1951) showed that the L forms of a number of amino acids disappeared from the lumen of the intestine of the rat more rapidly than the corresponding D-enantiomorphs, and they regarded this as evidence for the existence of a stereochemically specific mechanism for active absorption of L-amino acids. Wiseman (1953) showed that from solutions of a number of racemic amino acids the L-enantiomorphs could be transferred against a concentration gradient by the intestine *in vitro*, but that this did not occur with the D-enantiomorphs, and these results were confirmed by Agar, Hird & Sidhu (1953). Matthews & Smyth (1954) showed that the L-enantiomorphs of some amino acids appeared in the blood stream in larger quantities than the D-enantiomorphs when the racemic amino acid was present in the lumen of the intestine. All these results were in keeping with the view that transport of L-amino acids involves an active process, and it was assumed that this did not apply to D-amino acids. Further evidence, however, suggested that this view on D-amino acid transfer must be revised, since it was shown by Jervis & Smyth (1959*a*) that competition between some D- and L-amino acids was possible, and also by Jervis & Smyth (1959*b*) that the rates of absorption of both the D- and L-enantiomorphs of histidine and methionine were not proportional to their concentration in the intestine. The following experiments were therefore undertaken to obtain more evidence for the possible existence of an active mechanism for transfer of D-amino acids. A preliminary account has been given by Jervis & Smyth (1958).

METHODS

The D-amino acid chosen for study was D-methionine, partly because there is evidence that it appears to compete with L-histidine (Jervis & Smyth, 1959*a*) and also because it was desirable to use an amino acid for which a specific colorimetric method of estimation was available.

The general principle was to attempt to create experimental conditions where movement of D-methionine against a concentration gradient could be observed. It seemed possible that failures to achieve this in previous experiments were due to (1) the presence of L-methionine which competed for the mechanism and (2) the simultaneous movement of water. Fisher (1955) and Smyth & Taylor (1957) have shown that active movement of fluid

takes place in the intestine *in vitro*, and hence the relative rates of movement of solvent and solute will be an important factor in determining whether a substance can be concentrated. It was found that the necessary conditions could be achieved (*a*) by carrying out experiments where D-methionine was used without the L-form, (*b*) by using phosphate saline instead of bicarbonate saline, and (*c*) by using the ileum instead of the jejunum. It has been shown by Wilson & Wiseman (1954) and by Smyth & Taylor (1957) that these last two conditions reduce the rate of water transfer.

Male albino rats were used, and the intestinal preparation was the sac of rat everted small intestine as described by Wilson & Wiseman (1954). The details of the technique were as described by Parsons, Smyth & Taylor (1958), except that phosphate saline (Krebs, 1933) was used instead of bicarbonate saline. In making the sacs the small intestine below the duodenum was removed and divided into five approximately equal parts, and sacs were made from either the fourth or fifth segment counting from the jejunal end. The saline contained 500 mg glucose/100 ml. in all experiments and D-methionine was present initially in equal concentrations in the mucosal and serosal fluid. After shaking the sacs for 1 hr at 38° C the concentration of D-methionine was estimated in the mucosal and serosal fluids and the transfer of water and amino acid was calculated. The methods of calculating and expressing the results were those used by Parsons *et al.* (1958) with some small modifications. For the convenience of the reader the terms are defined as follows. The fluid in which the sacs are suspended is the mucosal fluid, the fluid inside the sac is the serosal fluid. The *mucosal fluid transfer* is the decrease in volume in mucosal fluid which occurs during the course of the experiment, the *serosal fluid transfer* is the increase in volume of serosal fluid. The *mucosal substrate transfer* is the amount of substrate (in this case D-methionine) which disappears from the mucosal fluid, the *serosal substrate transfer* is the increase in the amount of substrate in the serosal fluid; a decreased amount is described as negative serosal transfer. *Gut fluid uptake* and *gut substrate uptake* are the amounts of fluid or substrate not accounted for in the mucosal or serosal fluid. The difference between the final serosal and final mucosal concentrations is the *final concentration gradient*. The following additional terms are also used. The mucosal substrate transfer divided by the mucosal fluid transfer is the *mucosal concentration transferred*, and the serosal substrate transfer divided by the serosal fluid transfer is the *serosal concentration transferred*.

The transfer of methionine and fluid is expressed in amounts per unit weight of tissue. Parsons *et al.* (1958) used milligrams initial wet weight of tissue but this procedure gives figures which are inconveniently small, and therefore figures for transfer are given per gram initial wet wt.

Chemical. The D-methionine was obtained commercially, and freedom from contamination with L-methionine was tested by using L-amino acid oxidase from *Neurospora*. The D-methionine was estimated either colorimetrically by the method of McCarthy & Sullivan (1941) or enzymically with D-amino acid oxidase prepared from kidney according to the method of Bender & Krebs (1950).

RESULTS

Transfer of D-*methionine against a concentration gradient*

The results of a typical experiment with D-methionine are included in Table 1. In referring to this experiment it shall be understood that the figures given for D-methionine and fluid transfer refer to the amounts/g wet wt. of intestine. Initially D-methionine was present in equal concentrations (19·5 mM) in both mucosal and serosal fluids. During the course of the experiment 53·0 μmoles of D-methionine and 1·09 ml. fluid left the mucosal fluid, the D-methionine concentration in the fluid leaving the mucosal side

being 48·6 mM, i.e. greater than the concentration of 19·5 mM initially present in the mucosal fluid. Of the D-methionine and fluid leaving the mucosal side 33·0 μmoles of methionine and 0·50 ml. fluid were retained in the gut and the rest was transferred to the serosal fluid. The gut uptake of methionine was relatively greater than the gut uptake of water, and hence the fluid transferred to the serosal side contained a smaller concentration (33·8 mM) of methionine than that leaving the mucosal side. In spite of this it was still greater than the concentration initially present in the serosal side, and hence the final concentration (24·2 mM) in the serosal fluid was greater than the initial concentration. The total process resulted in D-methionine being moved against a concentration gradient.

TABLE 1. Transfer of D-methionine by sacs of rat everted intestine *in vitro*. In the experiments with inhibitors the initial concentration of KCN was 10^{-2} M and of 2:4-dinitrophenol 2×10^{-4} M. Duration of experiment 1 hr. Negative values of the final concentration gradient mean that the final serosal concentration is less than the final mucosal, and negative values for serosal transfer mean a loss from the serosal side

		In presence of KCN	In presence of 2:4-dinitrophenol
Initial wet wt. of sac (g)	0·84	0·90	1·04
D-methionine			
Initial mucosal and serosal concn. (mM)	19·5	19·2	20·4
Final mucosal concn. (mM)	17·6	18·8	20·0
Final serosal concn. (mM)	24·2	17·1	18·4
Final concentration gradient (mM)	6·6	−1·7	−1·6
Mucosal transfer (μmoles/g)	53·0	7·4	9·5
Serosal transfer (μmoles/g)	20·0	−4·6	−4·4
Gut uptake (μmoles/g)	33·0	12·0	13·9
Fluid			
Mucosal transfer (ml./g)	1·09	0·04	0·19
Serosal transfer (ml./g)	0·59	−0·07	−0·14
Gut uptake (ml./g)	0·50	0·11	0·33
Concentration of D-methionine transferred			
Mucosal (mM)	48·6	187·3	50·8
Serosal (mM)	33·8	67·6*	32·2*

* This is the concentration of fluid moved from the serosal side into the intestine.

In eight other experiments very similar results were obtained. In two further experiments the pattern was similar except that the serosal transfer of fluid was smaller, a relatively larger amount of the mucosal transfer being retained in the tissue. In these cases the concentration of D-methionine transferred to the serosal side was even greater than in the experiment in Table 1.

Effect of inhibitors

One method of demonstrating an active transport is by using inhibitors, and in the present experiments the inhibitors were 2:4-dinitrophenol and potassium cyanide. Two experiments were done with each inhibitor and one of each of these experiments is included in Table 1. It will be observed

that with the concentration used there was a marked inhibition of movement of both fluid and D-methionine. No serosal transfer of either took place and in fact solutions of D-methionine moved from the serosal fluid into the intestine.

The result was that the final concentration gradient was negative, i.e. the final serosal concentration was smaller than the final mucosal concentration. The high concentration of D-methionine transferred from the mucosal side in the presence of cyanide might occasion some surprise, suggesting that the D-methionine transfer mechanism was to some extent cyanide-resistant. This conclusion is not warranted, as is shown by the following consideration. Initially there is no D-methionine in the intestine. Even if the tissue is dead it is conceivable that a small amount of D-methionine may diffuse in from both sides. If the fluid movement is very small this may result in movement of a very high concentration of D-methionine. Examination of Table 1 shows in fact that a high concentration (67 mM) of D-methionine also diffuses into the intestine from the serosal side. There is thus no need to postulate any active transport of D-methionine in the presence of cyanide.

The effects of 2:4-dinitrophenol are also shown in Table 1 and are very similar to those obtained with potassium cyanide. The absolute amount of mucosal transfer was greatly reduced, methionine actually disappeared from the serosal side, and no movement against a concentration gradient took place.

Competition between D- *and* L-*enantiomorphs of methionine*

The previous sections offer evidence for an active movement of D-methionine when L-methionine is not present. If the views of Jervis & Smyth (1959*a*) on competition are correct it might be expected that even if the other conditions remained the same movement of D-methionine against a concentration gradient could no longer be shown in the presence of L-methionine. Experiments were carried out to test this point, in which the conditions were similar to those described in earlier sections, except that DL-methionine was used instead of D-methionine. In these experiments D-methionine could not be estimated colorimetrically and D-amino acid oxidase was used.

The results of an experiment of this type are shown in Table 2. This table includes results from both the fourth and fifth segments of intestine. In the fourth segment DL-methionine was present, in the fifth segment D-methionine only. It is apparent from these results that the presence of L-methionine had an inhibitory action on the transfer of D-methionine. No movement against a concentration gradient took place, and the absolute transfer was greatly reduced. In contrast to the reduction in methionine

transfer there was no obvious inhibition of fluid transfer, and the inhibition caused by L-methionine is thus more specific than that due to cyanide or dinitrophenol. Somewhat similar results were obtained in two further experiments, the only difference being that in both of these the inhibition of D-methionine transfer in the presence of L-methionine was even more striking, and the serosal D-methionine transfer was negative, i.e. some D-methionine disappeared from the serosal fluid. It will be observed that this also happened in the case of inhibition with 2:4-dinitrophenol and potassium cyanide.

TABLE 2. Competition between D- and L-methionine for intestinal transport by sacs of rat everted intestine. The sacs were made from the fourth and fifth segments of intestine. Duration of experiment 1 hr. Negative values of the final concentration gradient mean that the final serosal concentration is less than the final mucosal, and negative values for serosal transfer mean a loss from the serosal side. It should be noted that DL-methionine was present in sac 4, whereas only the D-enantiomorph was present in sac 5

Segment	Sac 4	Sac 5
Initial wet wt. (g)	0·78	1·05
Initial concn. of D-methionine (mM)	18·3	18·3
Initial concn. of L-methionine (mM)	20·0	—
D-methionine		
Final mucosal concn. (mM)	18·8	16·1
Final serosal concn. (mM)	11·6	25·6
Final concentration gradient (mM)	−7·2	9·5
Mucosal transfer (μmole/g)	19·1	48·5
Serosal transfer (μmole/g)	3·3	20·4
Gut uptake (μmole/g)	15·8	28·1
Fluid		
Mucosal transfer (ml./g)	1·52	1·07
Serosal transfer (ml./g)	1·02	0·53
Gut uptake (ml./g)	0·50	0·54
Concentration of amino acid moved		
Mucosal (mM)	12·5	45·4
Serosal (mM)	3·2	38·5

DISCUSSION

The results show that in the experimental conditions used D-methionine was transferred against a concentration gradient, and hence it would seem probable that there is an active mechanism involved in its transfer. This is supported by the fact that the transfer was abolished by 2:4-dinitrophenol and potassium cyanide, and by competition with L-methionine. It is also in agreement with the findings of Jervis & Smyth (1959*c*) that the rate of absorption of D-methionine, like that of L-methionine, is not proportional to the concentration present in the intestine. The competition between D- and L-forms of the same amino acid is of considerable interest in relation to the work of Jervis & Smyth (1959*a*) who found competition between L-methionine and D-histidine for intestinal absorption and to a lesser extent between L-histidine and D-methionine. The concept of

competition is now extended to competition between the enantiomorphs of the same amino acid. The effectiveness of L-methionine in competing with D-methionine is in keeping with the greater affinity of L-methionine for the transport mechanism, suggested by Jervis & Smyth (1959*c*) on the basis of investigations on the Michaelis constants in intestinal transport. It is also of interest to compare the results with those of Wiseman (1953), who first produced clear evidence of active transport of L-amino acids. In Wiseman's experiments racemic mixtures of amino acids were used and it was found that L-methionine but not D-methionine could be transferred against a concentration gradient. Our results confirm Wiseman's experimental observations, but suggest an interpretation of these which was not suspected at the time, i.e. that the failure to find active transport of D-methionine was in fact due to the presence of L-methionine. Wiseman (1955) also showed that there is a mechanism in the intestine for active transfer of amino acids for which a number of L-amino acids compete, and that L-methionine appears to have a greater affinity for the mechanism than the other L-amino acids tested. It appears from the present results and from those of Jervis & Smyth (1959*a*) that the same mechanism is capable of dealing with at least some D-amino acids, and the mechanism postulated by Wiseman (1955) is therefore not absolutely stereochemically specific. It should be stressed that the active transfer of D-methionine does not necessarily mean that all D-amino acids can be moved actively, and in fact preliminary experiments with D-histidine have not so far shown that this amino acid can be moved against a concentration gradient. The specificity of the mechanism could be such that it permits the transfer of many different L-amino acids, but being less favourable to the D configuration only permits the transfer of a smaller number of D-amino acids. Schemes for such a possibility have been suggested by Finch (1959).

The details of the mechanism of transfer of D-methionine still remain unknown. The inhibition by cyanide and by 2:4-dinitrophenol seemed at first to suggest an enzymic mechanism dependent on metabolic energy; but this does not necessarily follow, as the cyanide- and dinitrophenol-sensitive mechanisms may be involved only indirectly in transport, and may be more concerned with maintenance of the integrity of the cell, without which the transport mechanism cannot work. The preference of the mechanism for the L-enantiomorph, although also consistent with an enzymic mechanism, does not prove enzyme participation, as the stereochemical preference may be for sites on a non-enzymic carrier. On the other hand, competition between the D- and L-forms does not exclude direct participation of an enzymic mechanism. Although competition between two enantiomorphs of one substance for the same enzyme is unusual, there are other examples known of the action of an enzyme on one

enantiomorph being inhibited by the presence of the other enantiomorph, (Stahmann, Fruton & Bergmann, 1946; Massey, 1953), and there are also enzymes known which can act on both enantiomorphs of one substance. There is in fact an enzyme which will act on both L- and D-methionine (Kallio & Larson, 1955), but this particular enzyme is not likely to be involved in the present mechanism as it produces racemization, and the experiment quoted in Table 2, where the amino acid was estimated by D-amino acid oxidase, would seem to exclude this possibility. It must be concluded that the intestine possesses a mechanism for active transfer of both D- and L-methionine. The mechanism has a preference for the L-form, but it is not possible at present to suggest further details.

The results suggest that considerable care is necessary in deciding about the presence or absence of an active process in intestinal transfer, on the basis of movement or absence of movement against a concentration gradient. It is now well established that an active movement of water takes place in the intestine (Fisher, 1955; Smyth & Taylor, 1957) and the relative rates of water movement and solute movement must obviously be taken into consideration. The possibility of solutes being 'entrained' in an active water stream has been discussed by Fisher (1955), and the effects of 'solvent drag' have been discussed by Ussing and his co-workers (for references see Ussing, 1957). These considerations did not, however, include the possible case where solute and solvent are actively moved through a barrier by independent processes. It would seem possible in such cases that an active process concerned with solute movement might well be masked by one concerned with solvent movement. Thus, while achievement of concentration of a solute by the intestinal preparation can be regarded as evidence of the presence of a mechanism other than diffusion, failure to achieve a concentration is not necessarily evidence for the absence of such a mechanism. The relative rates of solvent and solute flow must be considered and conclusions should be made only in the light of such considerations.

SUMMARY

1. A study has been made of the transfer of D-methionine by everted sacs of ileum in conditions designed to reduce water transport.

2. Under these conditions D-methionine can be moved against a concentration gradient.

3. The movement against a concentration gradient is prevented by 2×10^{-4}M 2-4:dinitrophenol, 10^{-2}M cyanide and by the presence of L-methionine.

4. It is suggested that the transfer mechanism for L-methionine is not absolutely stereochemically specific, and that D-methionine is able to utilize this mechanism, although it has a lower affinity than L-methionine.

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EFFECT OF IONIC ENVIRONMENT ON INTESTINAL SUGAR TRANSPORT

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Glucose is absorbed from the intestine by two different mechanisms, diffusion and active transport. The former is effected by a concentration difference between the lumen and the blood; the latter proceeds against a concentration gradient. Active transport hastens the absorption up to a certain limit of concentration and causes the glucose to be completely absorbed even if the concentration in the lumen falls below that in the blood. Whereas the experimental approach to diffusion is relatively simple and clear-cut, active transport must be studied in 'living' preparations such as surviving isolated loops of intestine (Fisher & Parsons, 1949; Darlington & Quastel, 1953; Wilson & Wiseman, 1954). In such a preparation the sugar can be present in identical initial concentrations on both serosal and mucosal sides: an increase in concentration of sugar on either side, not accounted for through changes in volume, indicates that active transport is involved. This kind of experimental approach in mammalian gut has several drawbacks. One of them is the quantitative recovery of the sugar. This is complicated by the rapid metabolism of part of the glucose by the surviving gut tissue (Fisher & Parsons, 1950). Instead of glucose, however, 3-0-methylglucose (3-methylglucose) can be used. This sugar behaves like glucose in regard to intestinal absorption (Csáky, 1942), but since it is not metabolized (Csáky & Wilson, 1956; Csáky & Glenn, 1957), it can be quantitatively recovered. Another tiresome factor in connexion with the study of sugar transport in surviving mammalian gut is that the preparation has to be kept at body temperature and well supplied with oxygen. This difficulty can be eliminated by the use of the intestine of a poikilothermic animal, which transports both glucose and 3-methylglucose at room temperature and requires less vigorous oxygenation (Csáky & Fernald, 1960).

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This work was undertaken in order to study the effect of ionic environment on the active transport of 3-methylglucose from the small intestine. For this purpose a simple technique was developed for the study of active transport in the isolated intestine of the toad.

METHODS

Modified Ringer's solutions isosmotic with the plasma of frogs were used in this study (Boyle & Conway, 1941); the composition of the various solutions is shown in Table 1. The sugar was dissolved in these solutions to make concentrations as indicated in each type of experiment, but the starting concentration was always identical on both sides of the gut wall.

TABLE 1. Composition of media used in the experiments

		Final solutions made by mixing salt solutions on a volume basis					
Salt solutions	(m-mole/l.)	*A*	*B*	*C*	*D*	*E*	*F*
NaCl	116	85·0	—	—	—	—	—
KCl	117	2·1	2·1	2·1	2·1	2·1	2·1
$CaCl_2$	83	1·1	1·1	1·1	1·1	1·1	1·1
$MgSO_4$	185	1·2	1·2	1·2	1·2	86·2	1·2
$NaHCO_3$	112	1·5	1·5	1·5	1·5	1·5	1·5
Na_2HPO_4	124	1·0	1·0	1·0	1·0	1·0	1·0
NaH_2PO_4	124	2·2	2·2	2·2	2·2	2·2	2·2
LiCl	116	—	—	85·0	—	—	—
Na_2SO_4	97	—	85·0	—	—	—	—
Li_2SO_4	97	—	—	—	85·0	—	—
Choline Cl	116	—	—	—	—	—	85·0

Large toads (*Bufo bufo*) were decapitated and their spinal cords pithed. The small intestine was then removed and placed in the corresponding physiological solution after first rinsing through the lumen with a syringe. The gut was then mounted on the apparatus shown in Fig. 1. The volume of the mucosal compartment was 14 ml. and that of the serosal compartment 10 ml. At the beginning of the experiment and at certain intervals thereafter samples were taken from either mucosal or serosal compartments or both and the sugar content was analysed with the anthrone reagent (Mokrasch, 1954). The error of this analysis was $< 2\%$.

Phenol red in a final concentration of 10 mg/l. was routinely added to the mucosal compartment. This served as a convenient way to check 'leaks' and to calculate the movement of fluid, if any, from or to the mucosal compartment. Fluid movement was also determined at the end of each experiment by emptying the contents of both compartments and weighing the material. All experiments were carried out at room temperature ($19 \pm 2°$ C).

RESULTS

Fluid movement. In all experiments the movement of fluid was measured both with phenol red and by direct weighing at the end of the experiment. There was no measurable movement in any of the experiments described below.

Transport and recovery of glucose and 3-methylglucose. Table 2 shows the results of the experiments in which solution *A* was used on both mucosal and serosal sides. The initial concentrations of the two sugars were approximately the same on both sides, but after 4 hr the concentration

had decreased on the mucosal and in some cases increased on the serosal side. Only 72% of the glucose could be recovered at the end of the experiment. In the case of 3-methylglucose the recovery was better than 90%. Consequently, the increase in the serosal concentration was more consistent with 3-methylglucose than with glucose, although more glucose than 3-methylglucose disappeared from the mucosal compartment.

In all subsequent experiments the concentration of 3-methylglucose in the serosal compartment was measured. The rate of increase in the concentration was expressed as 'transport' in μmole/hr.

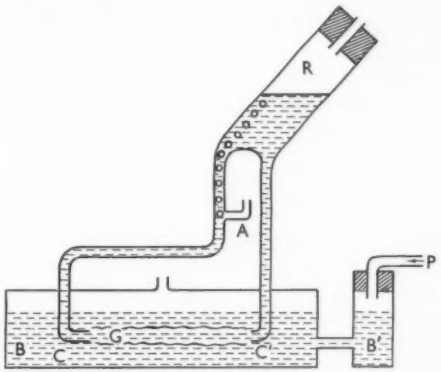

Fig. 1. Diagram of apparatus used in study of transport in surviving toad intestine. The gut (G) is mounted on cannulae C and C′ through which the mucosal surface is perfused from reservoir R. Air bubbles through opening A keep the contents in steady circulation. The serosal surface is in contact with the liquid in bath B, the content of which is gently agitated with a small respiratory pump P connected through container B′.

TABLE 2. Transport and recovery of glucose and 3-methylglucose in the surviving isolated small intestine of the toad

	Absolute amounts (mg)						
	0 hr			4 hr			Total recovery after 4 hr (%)
	Mucosal*	Serosal†	Total	Mucosal‡	Serosal‡	Total	
Glucose	5·80	4·18	9·98	3·34	3·64	6·98	69·9
Glucose	6·82	5·39	12·21	3·32	4·60	7·92	64·9
Glucose	7·58	5·23	12·81	4·98	5·64	10·62	82·9
						Mean	72·6
3-Methylglucose	7·52	5·28	12·80	6·33	6·28	12·61	98·5
3-Methylglucose	7·48	5·25	12·73	6·17	5·71	11·88	93·3
3-Methylglucose	6·89	5·23	12·12	5·29	5·84	11·13	91·8
						Mean	94·5

* In 14 ml. † In 10 ml. ‡ There was no measurable volume change in either compartment during 4 hr.

3-Methylglucose transport in the absence of sodium or chloride. Both mucosal and serosal surfaces were exposed to the same solutions (*A*, *B*, *C*, *E*, *F*). The results are summarized in Table 3. The replacement of chloride by sulphate (solution *B*) somewhat diminished the rate of active transport but did not completely abolish it. The replacement of Na by Li (solution *C*), Mg (solution *E*), or choline (solution *F*) completely abolished the active transport. There was no measurable active transport of xylose in solution *A*.

TABLE 3. Effect of the ionic composition of the medium on the transport of 3-methylglucose and xylose (4 and 6 hr experiments)

Solution	Principal cation	Principal anion	No. of experiments	Sugar transported (μmole/hr; mean ± S.D.)
		3-Methylglucose, 2·5 μmole/ml.		
A	Na	Cl	6	1·03 ± 0·38
B	Na	SO_4	4	0·41 ± 0·19
C	Li	Cl	5	0·0
E	Mg	SO_4	4	0·0
F	Choline	Cl	4	0·0
		Xylose, 2·5 μmole/ml.		
A	Na	Cl	4	0·0

TABLE 4. 3-Methylglucose transport in solution *C* followed by solution *A* in the same loop of intestine. Initial 3-methylglucose concentration, 2·5 μmole/ml.

	Solution (see Table 1)	Principal cation	Principal anion	Sugar transported (μmole/hr)
Expt. 1	*C*	Li	Cl	0·0
	A	Na	Cl	0·79*
Expt. 2	*C*	Li	Cl	0·0
	A	Na	Cl	0·92*

* There was no measurable transport during the first hour following the change of solution.

Reversibility of inhibition due to lack of sodium. The gut was placed first in solution *C* for 2 hr, during which time no sugar transport was observed. It was then transferred into another bath with solution *A* on both serosal and mucosal sides. There was a complete reversal of the inhibition, showing that the absence of Na did not permanently damage the sugar-transporting mechanism of the mucosa (Table 4).

Effects of lack of sodium on mucosal and serosal sides. The following experimental procedure was adopted. In one series of experiments the intestine was placed in solution *D*, which was in contact with the serosal surface, and solution *A* circulated through the lumen of the intestine. In another series of experiments the situation was reversed. The results shown in Table 5 indicate that when Na-containing solution was in contact

with the mucosal surface, there was an active sugar transport regardless of the composition of solution on the serosal side; if the serosal surface was in contact with the Na-containing solution and the mucosal side bathed with the Li-containing solution, there was no measurable transport of 3-methylglucose.

TABLE 5. Transport of 3-methylglucose with solutions of different ionic composition on serosal and mucosal sides of the intestine (4 hr experiments). Initial 3-methylglucose concentration: 2·5 μmole/ml.

Solution		Principal cation	Principal anion	No. of experiments	Sugar transported (μmole/hr; mean ± S.D.)
D	Mucosal	Li	SO_4	3	0
B	Serosal	Na	SO_4		
B	Mucosal	Na	SO_4	6	0·63 ± 0·34
D	Serosal	Li	SO_4		

DISCUSSION

There was no appreciable net water transport in the preparations studied in this work. Since the original sugar concentration was approximately the same in the mucosal and serosal compartments, the progressive concentration changes, by definition, indicate an active transport of 3-methylglucose from the mucosal into the serosal compartment. Xylose, which is not actively transported from the mammalian gut, was not transported in unmodified Ringer's solution. The recovery of 3-methylglucose was better than 90%.

The value of this method in studying active sugar transport is somewhat limited, inasmuch as experiments *in vitro* in general produce a situation different from that in the live animal. *In vitro*, the sugar molecule passes through the epithelial layer and is transferred into a compartment composed of connective tissue, muscle and serosal mesothelium. In the live animal the capillaries reach to the submucosal layer; thus the second compartment is eliminated. The significance of the compartment occupying the anatomical space between the mucosal epithelium and the 'free' serosal compartment is established by the finding that about 10% of the 3-methylglucose cannot be recovered in the combined 'free' compartments on mucosal and serosal sides. However, this problem was not further pursued in this study.

It has been repeatedly shown that changes in ionic environment profoundly influence the intestinal glucose absorption both *in vitro* and *in vivo*. Sodium, potassium, calcium, and magnesium ions were found to be necessary for 'normal' absorption. The pertinent literature was summarized recently by Riklis & Quastel (1958). The same authors demonstrated that increasing the potassium concentration to 15·6 m-equiv/l. on both mucosal

and serosal sides stimulates the transfer of glucose across the surviving guinea-pig intestine. When all the sodium on both sides was replaced by potassium there was practically no transfer of glucose, indicating that sodium ions are essential for glucose transfer.

The results of the present investigation indicate that sodium ions are essential for active sugar transport across the intestine. This is apparent from the observation that replacement of sodium by lithium, magnesium or choline completely inhibited active transport of 3-methylglucose. This inhibition is reversed by sodium, showing that the replacement of sodium did not permanently damage the epithelium. Moreover, it is apparent from the data presented here that sodium is essential only on the mucosal surface.

No attempt was made to investigate the concentration of sodium which is essential for active sugar transport. In unmodified Ringer's solution the bulk of the cations are in the form of sodium. Although the bulk of sodium was replaced in these experiments, this ion was still present in a concentration of 7·3 m-mole/l. in the media containing lithium, magnesium or choline as the principal cations (see Table 1). The lack of active sugar transport in these solutions indicates that more than 7·3 m-mole/l. of sodium is necessary for the sugar-transporting function.

Good evidence was recently presented by Curran & Solomon (1957) that sodium is actively transported in the gut from the mucosal to the serosal side. The nature of a sodium 'pump' was recently described in the frog skin by Koefoed-Johnsen & Ussing (1958). According to their concept the outward-facing membrane of the frog's skin epithelium is permeable to sodium, whereas the inward-facing membrane is practically impermeable to free sodium ions but sodium is 'pumped' across this membrane by an active process. The data presented here indicate that the intestinal sugar 'pump' does not function in the absence of sodium on the mucosal side.

SUMMARY

1. A method is described for the study of active sugar transport in the surviving isolated intestine of the toad by using the non-metabolized 3-methylglucose.

2. In unmodified frog Ringer's solution 3-methylglucose is actively transported from the mucosal to the serosal side.

3. Replacement of sodium by lithium, magnesium or choline abolishes active sugar transport. This effect is reversible if the original sodium concentration is restored.

4. Sodium is essential for active sugar transport on the mucosal but not on the serosal side.

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THE PASSAGE OF THYROXINE-BINDING SUBSTANCES BETWEEN MOTHER AND FOETUS IN PREGNANT RABBITS

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From a study of the behaviour of radioactive thyroxine added to foetal and maternal serum from pregnant rabbits, Osorio & Myant (1958) concluded that rabbits have two distinct thyroxine-binding substances in the blood. One, present in the serum of adults and found in foetal serum only at the end of pregnancy, moves on electrophoresis in a barbiturate buffer to a position between albumin and the α_1-globulin. The other, present in the serum of foetuses and new-born rabbits, moves between the α_2- and β-globulins. The binding substance in maternal serum was called 'adult thyroxine-binding protein' (adult TBP), and the other was called 'foetal thyroxine-binding protein' (foetal TBP), although there was no direct evidence that either substance was a protein.

In some of our observations on maternal serum, a small peak of radioactivity was present between the α_2- and β-globulins (Myant & Osorio, 1959). This suggested that some foetal TBP may reach the maternal circulation. In most of these experiments the amount of labelled thyroxine added to serum was at least 5 μg/100 ml. It was shown that although the binding affinity of foetal TBP is high, its binding capacity—defined as the total number of binding sites per unit volume of serum—is much less than that of maternal TBP. If, therefore, thyroxine is added to serum containing foetal and maternal TBP, the proportion bound to foetal TBP will fall as the concentration of thyroxine is increased, since the binding sites on foetal TBP are saturated before those on maternal TBP. It thus seemed possible that clearer evidence for the presence of foetal TBP in maternal serum might be obtained by studying the binding of thyroxine at lower concentrations. Accordingly, we have studied the binding by maternal serum of radioactive thyroxine of high specific activity.

Another question raised by our previous work was the source of the adult TBP in the serum of foetal and new-born rabbits. From the evidence available it was impossible to decide whether the adult TBP which appeared in the foetal serum at about the 28th day was derived

from the maternal circulation, or whether the foetus itself had begun to synthesize it. In this paper we describe experiments which suggest that some of the adult TBP in the foetal circulation is derived from the mother.

METHODS

The rabbits were of the Dutch strain. Blood was obtained by cardiac puncture. Milk was obtained by means of a glass suction pump fitted over the rabbit's nipple. The casein was precipitated by acidification to pH 4·5 with a few drops of acetic acid and removed by centrifugation. Endogenous thyroxine in the circulation was labelled by giving the rabbit an injection of 200–300 μc of radio-iodide 30 hr before the blood sample was taken. The specific activity of the exogenous thyroxine (Abbott Laboratories Pacific Ltd., Chicago) varied from 20 to 30 μc/μg. After the addition of radioactive thyroxine to the serum or milk, the samples were allowed to stand at room temperature for at least 2 hr before the electrophoresis was begun. Paper electrophoresis was carried out in a barbiturate buffer at pH 8·6 by methods described elsewhere (Myant & Osorio, 1959). The serum was put on to the paper strip half way between the anode and the cathode, since this was found to increase the separation between albumin and the α-globulins. The distribution of radioactivity at the end of the electrophoresis was found by passing the dried paper through a continuously recording scanner. The papers were then stained with lissamine green and differentiated by washing with 5% acetic acid.

RESULTS

Foetal TBP in maternal serum

Exogenous thyroxine. Radioactive L-thyroxine (about 1 μg/100 ml. of serum) was added to the serum and the distribution of radioactivity was determined after separation of the proteins by paper electrophoresis.

A few preliminary observations were made on non-pregnant rabbits. In serum from adult males and females, the main peak of radioactivity was at the position of adult TBP, with a secondary peak at the rear of the albumin. In a few samples, from males and females, a small peak was just detectable between the α_2- and β-globulins (Fig. 1*a*), corresponding exactly to the position of the main peak of radioactivity found in foetal serum (Osorio & Myant, 1958).

In serum taken from pregnant rabbits the distribution of radioactivity was the same as that in non-pregnant rabbits until about the 21st day of pregnancy. At the 23rd day the peak at foetal TBP was always present and was usually about a third as high as the main peak at adult TBP (Fig. 1*b*). As the period of gestation increased, the peak at foetal TBP increased at the expense of that at adult TBP, until at the end of pregnancy the two peaks were nearly equal in height (Fig. 1*c*). After parturition the peak at foetal TBP decreased rapidly (Fig. 1*d*, *e*), and by the second week was as inconspicuous as in the serum of male or virgin female rabbits.

Some of the observations on the serum of pregnant rabbits were repeated with higher concentrations of thyroxine. Figure 1*f* shows the results of an

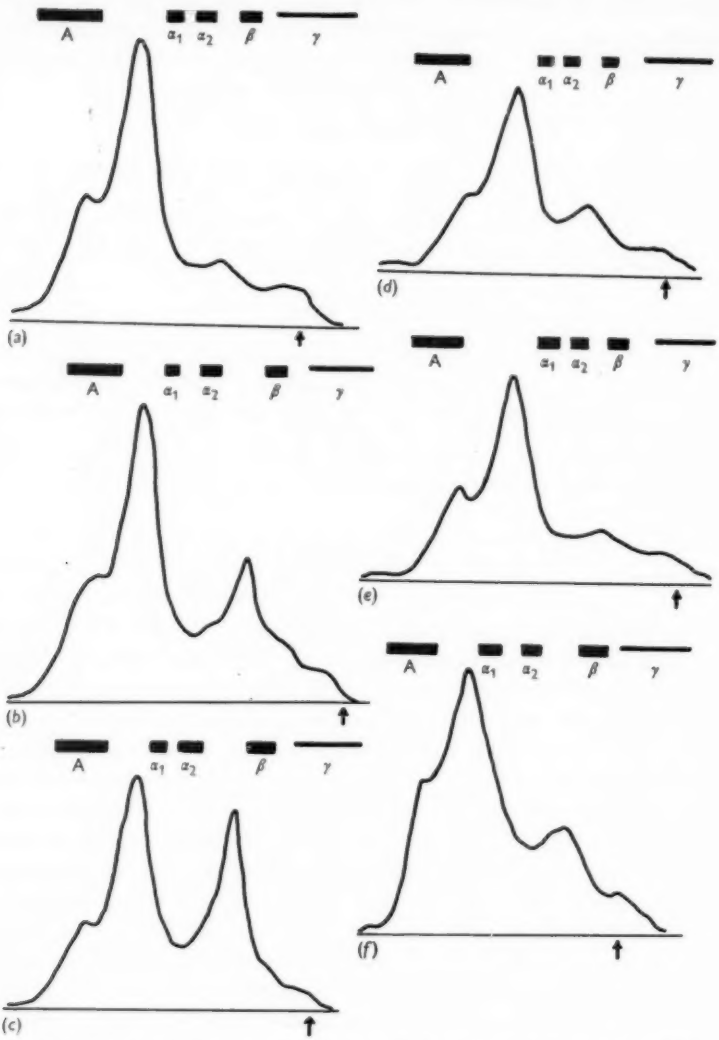

Fig. 1. Distribution of radioactivity along electrophoresis papers after addition of 1 μg ^{131}I-labelled thyroxine/100 ml. to serum from female rabbits; *a*, adult virgin; *b*, 23 days pregnant; *c*, the same rabbit as *b*, 28 days pregnant; *d*, the same as *b*, 1 day after parturition; *e*, the same as *b*, 11 days after parturition; *f*, the same serum as *c*, but with the addition of 5 μg radioactive thyroxine/100 ml. Position of protein bands shown above each curve (A = albumin). Arrow indicates line of application of serum.

experiment in which 5 μg of radioactive thyroxine/100 ml. serum was added to the same serum as that shown in Fig. 1*c*. At the higher concentration of thyroxine the proportion of the total radioactivity bound by foetal TBP was much reduced, as is shown by the diminution in the peak between the α_2- and β-globulins.

We have shown elsewhere that the binding capacity of TBP is related to the thyroxine concentration at which radioactivity is displaced from the main peak on the electrophoresis paper (Myant & Osorio, 1959, Text-fig. 4*a*). In this investigation observations were made on the binding capacity of adult TBP in the mother's serum at different stages of pregnancy. During the course of pregnancy there was no significant change in the concentration of thyroxine required to displace a given proportion of the radioactivity bound by adult TBP.

Endogenous thyroxine. When the endogenous thyroxine in the serum of non-pregnant rabbits was labelled, the distribution of radioactivity was similar to that observed with 1 μg of exogenous radioactive thyroxine/100 ml., except that the peak at albumin tended to be higher. As in the experiments with exogenous thyroxine, the peak at foetal TBP was either absent or only just detectable. When pregnant rabbits were injected with radio-iodide at the 24th day of gestation, the concentration of radioactive thyroxine in the mother's serum at 30 hr after the injection was much lower than that in the foetal serum and was not high enough to give clearly defined peaks with the recording scanner. It was therefore impossible to determine the distribution of endogenous radioactive thyroxine in maternal serum.

Adult TBP in the serum of new-born rabbits

When thyroxine-binding power is tested with radioactive thyroxine at a concentration of 5 μg/100 ml., adult TBP cannot be detected in foetal serum until a day or two before birth (Myant & Osorio, 1959). If new-born rabbits are suckled by their mother, the ratio of the peak of activity at adult TBP to that at foetal TBP rises gradually from the first day of birth until the third or fourth week. At this time the pattern of radioactivity resembles that in an adult rabbit.

A fortuitous observation suggested that the rise in adult TBP during the first few days of life does not occur if the new-born rabbit is separated from its mother. Experiments were done to test this possibility. Litters of new-born rabbits were divided into two groups at various ages, usually at the seventh day. One group was left with the mother. The others were kept in a separate cage and fed with cow's milk. Blood samples were then taken serially from both groups of rabbits and tested for thyroxine-binding power. Radioactive thyroxine at a concentration of 5–10 μg/100 ml. was added to the serum and the distribution of radioactivity measured after

paper electrophoresis. Whereas the peak of activity at adult TBP increased from day to day in the controls kept with their mother, in the others it fell rapidly and had almost disappeared by the second day of feeding with cow's milk (Fig. 2*a*, *b*). The fall in the peak at adult TBP was accompanied by a rise in the peak at foetal TBP. Similar, though less marked, effects were also observed when feeding with cow's milk was begun on the twelfth

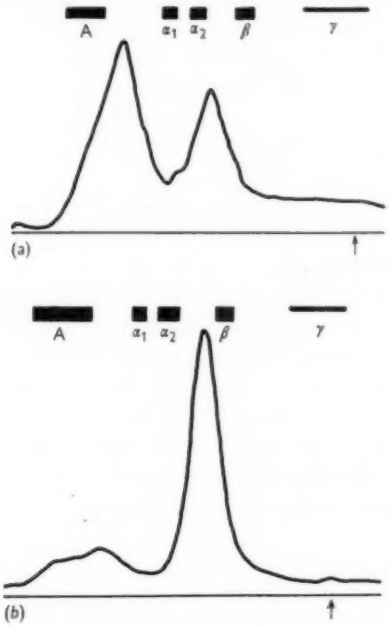

Fig. 2. Distribution of radioactivity along electrophoresis paper after addition of 5 μg labelled thyroxine/100 ml. to serum from *a*, 7-day-old rabbit; *b*, the same rabbit aged 9 days. The rabbit was kept with its mother until 7 days old, and was then removed and fed with cow's milk until it ate solid food.

day after birth. If the feeding with cow's milk was continued until the rabbit began to eat solid food (when about 1 month old) the peak at adult TBP, after its initial diminution, began to increase again until by the fifth week of life the pattern of radioactivity was similar to that in litter-mates suckled by the mother. Although the rabbits fed with cow's milk gained weight, they did not do so at the same rate as the normal litter-mates; at the end of the experimental period their weight was usually about 85% of that of the controls.

Adult TBP in milk

These results suggested that the adult TBP appearing in the serum of new-born rabbits is derived from the mother, to a considerable extent during the first 2 weeks, and to a smaller extent during the remaining period of suckling. This could be explained most easily if adult TBP were secreted in the milk. To find out whether this is the case, a sample of milk was obtained from a lactating rabbit. After removal of the casein, radioactive thyroxine (1 μg/100 ml.) was added to the milk and a sample put on paper for electrophoresis. Staining with lissamine green showed three bands of protein (Fig. 3*b*). In some cases there was also a band at the origin. When the paper was tested for radioactivity, a sharp peak was found in the clear zone between the two foremost bands of protein (Fig. 4*c*). The peak coincided with the zone between the albumin and α_1 bands seen

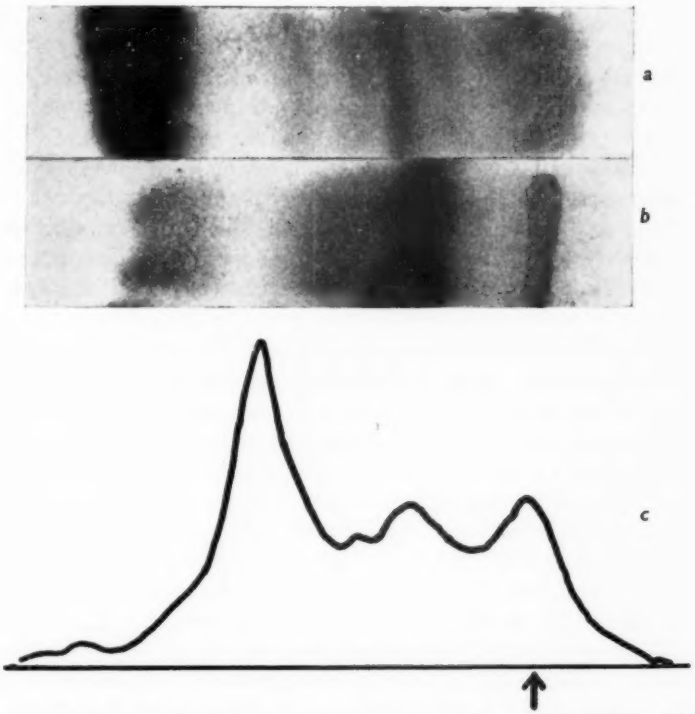

Fig. 3. Paper electrophoresis of *a*, serum, and *b*, milk from a lactating rabbit (samples run in parallel on same strip of paper and stained with lissamine green); *c*, distribution of radioactivity along *b* (1 μg radioactive thyroxine added/100 ml. of milk after removal of casein).

in a sample of the same rabbit's serum run in parallel on the same strip of paper (Fig. 3*a*). Smaller peaks of radioactivity were also present at the origin and at the main band of protein.

DISCUSSION

Foetal TBP in maternal serum

When serum containing radioactive thyroxine is analysed by paper electrophoresis, the distribution of activity along the paper is the result of a competition for thyroxine between the various thyroxine-binding substances present. The amounts of activity at the peaks corresponding to adult and foetal TBP are, therefore, only indirectly related to their concentrations in the serum. It was pointed out by Myant & Osorio (1959) that when thyroxine at low concentrations is shared between two or more binding substances the amount bound by each is proportional to the product of its binding affinity and its binding capacity. Since binding affinity is unlikely to alter under physiological conditions, any change in the distribution of radioactivity between adult and foetal TBP may be assumed to be due to a change in the binding capacity of one or other substance.

The results obtained with exogenous thyroxine show that foetal TBP is not usually detectable in the serum of a non-pregnant adult rabbit, though a small amount occasionally persists into adult life. During pregnancy foetal TBP always appears in the maternal serum and disappears again after parturition. From the relationship discussed above it follows that the rise in the peak of radioactivity at foetal TBP (Fig. 1*b*, *c*) could be caused either by a rise in the binding capacity of foetal TBP, or by a fall in that of adult TBP. Since the binding capacity of adult TBP does not decrease during pregnancy, the change in the pattern of radioactivity occurring towards the end of pregnancy must be caused by a rise in the binding capacity of the foetal TBP which appears in the mother's serum. The rise is due, presumably, to an increase in the concentration of foetal TBP, rather than to a change in the number of binding sites per molecule.

Although foetal TBP appears in the mother's serum during the second half of pregnancy and then increases in concentration until parturition, it never reaches the concentration we have observed in foetal serum. This is shown by the effect of a change in thyroxine concentration on the distribution of radioactivity in foetal and maternal serum. A peak of radioactivity at foetal TBP is still visible in the serum of 25-day-old foetuses, tested in the presence of maternal serum, when the concentration of added thyroxine is raised to 40 μg/100 ml. (Myant & Osorio, 1959, Text-fig. 4*c*). At a concentration of 5 μg/100 ml. the foetal TBP in maternal serum is

almost completely saturated, as shown by a marked diminution in the peak of radioactivity (Fig. 1*c*, *f*, this paper). The reason why the foetal TBP in maternal serum binds so much thyroxine at low concentrations of thyroxine is that its affinity for thyroxine is several times that of adult TBP.

The fact that the concentration of foetal TBP is higher in foetal than in maternal serum throughout pregnancy suggests that the foetal TBP in the mother is derived from the foetus. The rapid disappearance of foetal TBP from the mother's circulation after parturition is also consistent with this. If TBP is a protein, this explanation would make it necessary to assume that a protein can pass between mother and foetus at least as early as the 23rd day of pregnancy. However, there is no direct evidence that TBP is, in fact, a protein. In any case, it is well known that homologous proteins can cross the barrier separating the mother from the foetus in rabbits (Brambell, 1958). It should be pointed out that none of our observations excludes the possibility that foetal TBP arises independently in the mother and the foetus, or that it is made in the placenta and passes thence into the maternal and foetal circulations.

Adult TBP in the serum of the new-born rabbit

The effect of removing suckling rabbits from their mother can be explained by supposing either that the milk contains a factor necessary for the synthesis of a normal quantity of adult TBP during the first few days of life, or that it supplies the new-born animal with adult TBP itself. The failure of new-born rabbits to gain weight at the normal rate when fed with cow's milk, as given in these experiments, suggests that this diet is generally deficient. If new-born rabbits synthesize any of their own adult TBP, a deficient diet might contribute to the fall in the concentration of adult TBP in their serum when rabbit's milk is replaced by cow's milk. However, the presence in rabbit's milk of a thyroxine-binding substance with an electrophoretic mobility similar to that of adult TBP in serum is also consistent with the second possibility—that some, at least, of the adult TBP in the serum of new-born rabbits is normally derived from the mother. If adult TBP is a protein, this raises the difficulty of explaining how it reaches the blood of the suckling rabbit without being digested in the gut. Although it is known that in many species homologous proteins can be absorbed unchanged from the stomach during the first few days of life, attempts to demonstrate this in rabbits have not been successful (R. Halliday, personal communication). If the new-born rabbit cannot synthesize all its own adult TBP, it is reasonable to assume that this is also the case in the foetus, and that some of the adult TBP appearing in foetal serum towards the end of pregnancy is derived from the mother.

The presence of adult TBP in rabbit's milk might be expected to lead to the secretion of thyroxine in the milk. The amount of thyroxine secreted would depend on the relative concentrations of binding substance in milk and serum. Further, if the binding substance in milk is synthesized in the mammary gland, the concentration of thyroxine in the milk would also depend on the time available for equilibration between the thyroxine in the milk and the serum. We have no information on any of these points, nor is there any direct evidence about the extent to which the plasma thyroxine reaches the milk in lactating rabbits. However, Potter, Tong & Chaikoff (1959) have shown that radioactive thyroxine injected into lactating rats appears in the milk.

SUMMARY

1. The thyroxine-binding substance present in rabbit foetal serum (foetal TBP) is detectable in the maternal serum at the 23rd day of pregnancy and increases in concentration until parturition. After parturition, foetal TBP disappears from the mother's serum within a few days.

2. When new-born rabbits are suckled by their mother, the serum concentration of the binding substance present in adults (adult TBP) increases until it completely replaces the foetal TBP present at birth. If the new-born rabbits are fed with cow's milk instead of rabbit's milk, adult TBP disappears from their serum.

3. Rabbit's milk contains a thyroxine-binding substance with an electrophoretic mobility similar to that of adult rabbit's TBP.

4. It is suggested that foetal TBP reaches the mother's blood from the foetus and that the adult TBP in the serum of new-born rabbits is derived partly from the mother's milk.

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MECHANISM OF THE AFTER-POTENTIAL PRODUCTION IN THE GIANT AXONS OF THE COCKROACH

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There is evidence supporting the view that the spike potential of the cockroach giant axon is produced by an entry of sodium which is followed by an exit of potassium (Yamasaki & Narahashi, 1958, 1959*a*), as in the giant axon of the squid (Hodgkin, 1957). The spike potential of the cockroach axons is followed by a positive phase and a negative after-potential. The positive phase has been shown to be explicable on the assumption that the potassium conductance increases during the latter half of an action potential and does not at once return to its resting value, thereby causing the membrane to be hyperpolarized (Yamasaki & Narahashi, 1959*a*). Little is known about the processes underlying the production of the negative after-potential, although the mechanism has been suggested to be quite different from that of the positive phase (Yamasaki & Narahashi, 1959*b*). There is some evidence for other kinds of nerves that the negative after-potential is produced by an accumulation of potassium released in the immediate vicinity of the nerve membrane (Frankenhaeuser & Hodgkin, 1956; Greengard & Straub, 1958; Shanes, 1949*a*, 1951).

The present investigation was carried out in order to elucidate the mechanism of the negative after-potential production in the cockroach axon. Since the spike potential and the positive phase were thought to correlate closely with the negative after-potential, they also were subjected to analysis. The behaviour of these potentials was observed under a variety of external concentrations of potassium, because of the possible role of potassium in the production of the negative after-potential. The effect of calcium on these potentials was also examined, because of its well-known ability to affect the negative after-potential as well as excitability (see reviews by Brink, 1954; Shanes, 1958).

METHODS

The giant axons in the abdominal nerve cord of the American cockroach, *Periplaneta americana* L., were used throughout the experiments.

The methods of stimulation and recording were essentially similar to those described in

our previous papers (Yamasaki & Narahashi, 1959*a*, *b*). Stimulation was effected by a pair of silver-wire electrodes, while recordings were made by a micro-electrode inserted in an axon. The method, however, was modified in the following two respects. (1) The Ag-AgCl ground electrode was connected to the bath via a tube filled with 3 M-KCl solution and a Ringer-gelatine bridge. (2) For changing the bathing solution, the new solution flowed into the nerve chamber through an inlet tube, while the old solution was continuously sucked out through an outlet tube which opened just above the surface of bathing solution. By this method the surface of the bathing solution could be kept at a constant level during perfusion, and the micro-electrode could be kept inserted in the axon without causing appreciable damage. The amount of test solution necessary for a complete replacement was checked by using a solution containing a dye.

The Ringer's solution was the same as that described in the previous paper (Yamasaki & Narahashi, 1959*a*), and had the following composition (mM): Na^+, 214; K^+, 3·1; Ca^{2+}, 1·8; Cl^-, 216·9; $H_2PO_4^-$, 0·2; HPO_4^{2-}, 1·8; pH 7·2. The potassium concentration was changed between 0 and 10 mM by replacing Na by K, thus keeping the tonicity constant. The effects of varying the sodium concentration over such a small range should be negligible. When the calcium concentration was to be changed, $NaHCO_3$ was used as a buffer in place of phosphates, because high concentrations of calcium caused phosphates to be precipitated. In this case, the sodium and potassium concentrations were kept constant to avoid any effects of changing [Na], because the effect of [Ca] was examined over a wider range of concentrations than potassium. All the experiments were conducted at room temperatures ranging from 16·5 to 26·5° C.

RESULTS

Normal resting and action potentials

The resting potential of normal axons was in the range of 70–83 mV, with a mean of $77 \pm 0{\cdot}7$ mV (S.E. of mean, 24 measurements). The magnitude of the action potential was in the range of 88–110 mV, with a mean of $99 \pm 1{\cdot}4$ mV (19 measurements). The resting and action potentials are higher by 7 and 4 mV, respectively, than the values reported previously (Yamasaki & Narahashi, 1959*a*). It seems reasonable to assume that the values obtained in the present experiments are more reliable because of improved technique and also because of more critical selection of material.

The spike potential was followed by a positive phase, which was in turn followed by a negative after-potential. The magnitudes of the positive phase and of the negative after-potential varied considerably in different axons, the mean of the positive phase being 4·6 mV with a range of 1·2–9·0 mV, and the mean negative after-potential being 1·5 mV with a range of 0·35–3·0 mV.

Spike potential and positive phase

Effect of changing the external concentration of potassium. An example of a series of records is illustrated in Fig. 1. Records were taken at external potassium concentrations of 0, 3·1 (normal) and 10·0 mM. It will be seen that the positive phase decreased in height when the potassium concentration was raised from 3·1 to 10·0 mM and increased when the axon was

deprived of potassium. The resting potential and the spike potential were also decreased in magnitude by raising potassium and increased by lowering potassium. These changes are in agreement with those in squid giant axons (Hodgkin & Katz, 1949; Frankenhaeuser & Hodgkin, 1956).

Changes in magnitude during repetitive excitation. Figure 2*B* shows records taken with varying frequencies of repetitive stimulation at the

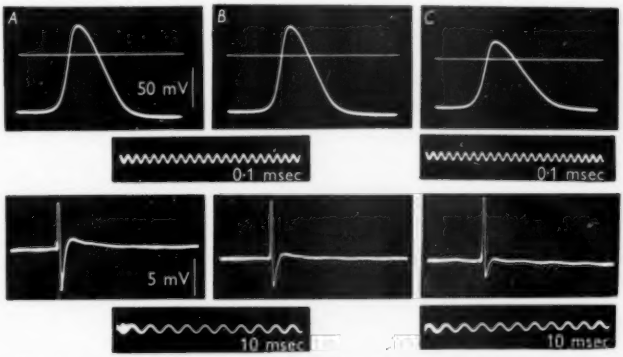

Fig. 1. Records of the action potential showing the effect of changing the external concentration of potassium. The upper records show the spike potential; zero potential level is indicated by a horizontal line. The lower records show the positive phase and the negative after-potential with higher amplification and slower sweep, the spike potential being too large to be recorded. *A*, in 0 mM-K; *B*, in 3·1 mM-K; *C*, in 10·0 mM-K. Temperature 19·5° C.

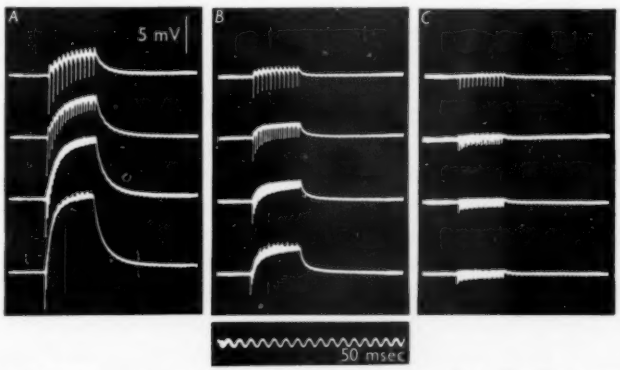

Fig. 2. Trains of impulses at varying frequencies recorded in different concentrations of potassium. Only the positive phase and the negative after-potential are seen; the spike potential is too large to be recorded. *A*, in 0 mM-K; *B*, in 3·1 mM-K; *C*, in 10·0 mM-K. Frequencies of impulses are, from top to bottom in each column, 50, 100, 150 and 200/sec. The same axon as in Fig. 1. Temperature 19·5° C.

normal external potassium concentration of 3·1 mM. The amplification used was too large to record the spike potentials. The membrane potential at the peak of the positive phase, measured relative to zero potential, decreased progressively during a train of impulses. A decrease was also seen when the measurement was made from the starting level for the preceding impulse. The higher the frequency, within limits, the greater was the decrease in magnitude. These changes in the positive phase were more prominent in a potassium-free solution, and were less marked in a potassium-rich solution (Fig. 2*A* and *C*). The spike height also decreased nearly exponentially during a train of impulses.

In the giant axons of the cockroach the positive phase is caused by a delayed and sustained increase in potassium conductance (Yamasaki & Narahashi, 1959*a*), as it is in squid giant axons (Hodgkin & Keynes, 1955; Frankenhaeuser & Hodgkin, 1956). Since the positive phase of the cockroach giant axons was found, as just described, to decrease with raised potassium concentration, it seems reasonable to suggest that the decrease in positive phase during a train of impulses is caused by an accumulation of potassium released during the latter half of each action potential. It follows that the negative after-potential is also likely to be due to the accumulation of the released potassium, because the increase in external potassium concentration would cause the nerve membrane to become temporarily depolarized (see also Yamasaki & Narahashi, 1959*a*). This hypothesis for the production of the negative after-potential will be analysed further in the following section.

Negative after-potential

Decay of negative after-potential. The time course of the decay of a negative after-potential was plotted on a logarithmic scale against time (Fig. 3). It is seen that the negative after-potential following a single impulse decayed exponentially. The time constant of decay ranged from 5·8 to 12·9 msec in different axons, with a mean of 9·2 msec.

The space constant of the cockroach giant axons was estimated as 0·86 mm (Yamasaki & Narahashi, 1959*b*), and the velocity of conduction as 6–7·2 m/sec (Boistel & Coraboeuf, 1954; Roeder, 1948). Hence it takes only about 0·1 msec for an impulse to conduct over a distance of one space constant. This means that, in so far as the slow phase of the action potential is concerned, the conditions are certainly close to those obtained with uniform polarization. Therefore, any declining phase of the action potential would decay exponentially if that phase were passive in nature (cf. Hodgkin & Rushton, 1946). For these reasons, it is probable that the negative after-potential following a single impulse in the cockroach giant axon decays passively.

The decay of the negative after-potential after cessation of repetitive stimulation at various frequencies was plotted on a logarithmic scale, as illustrated in Fig. 4. The decay was not linear over the whole time course. The initial brief period could be expressed as an exponential term with a time constant slightly longer than that for the decay after a single action potential. The time constant increased slightly when the stimulus frequency was increased. The terminal slow phase of the decay could also

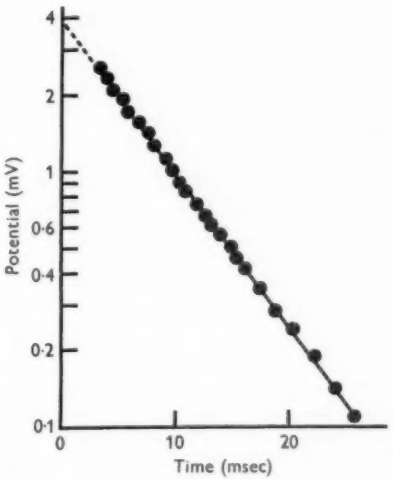

Fig. 3. Time course of the decay of the negative after-potential following a single impulse recorded from an axon bathed in normal Ringer's solution. The ordinate represents the potential on a logarithmic scale, and the abscissa is the time after the end of the spike. The straight line, drawn through the points by eye, corresponds to a time constant of 7·1 msec. Temperature 19·5° C.

be expressed as an exponential term, whose time constant varied over a wide range in different axons, sometimes exceeding 500 msec. In any particular fibre the time constant of the terminal slow phase depended on the frequency of stimulation, increasing with a rise in frequency.

Build-up of negative after-potentials on repetitive stimulation. On repetitive stimulation there appeared a progressive exponential build-up of the negative after-potential which finally attained a steady level. The magnitude of the steady level depended on the frequency of stimulation. The higher the frequency of stimulation, within limits, the higher was the level reached (Fig. 2).

The negative after-potentials would add in a linear manner during a train of impulses, if the resting potential varied with the external potassium concentration and the negative after-potential was attributable to the

accumulation of the released potassium during activity. This proved to be the case for squid giant axons and rabbit non-myelinated nerve fibres. (Frankenhaeuser & Hodgkin, 1956; Greengard & Straub, 1958). To make this point clear for the cockroach axons, the time courses of the three successive negative after-potentials together with the eighth after-potential, during a train of impulses at a frequency of 50/sec, were plotted on a logarithmic scale against time, as is shown in Fig. 5.

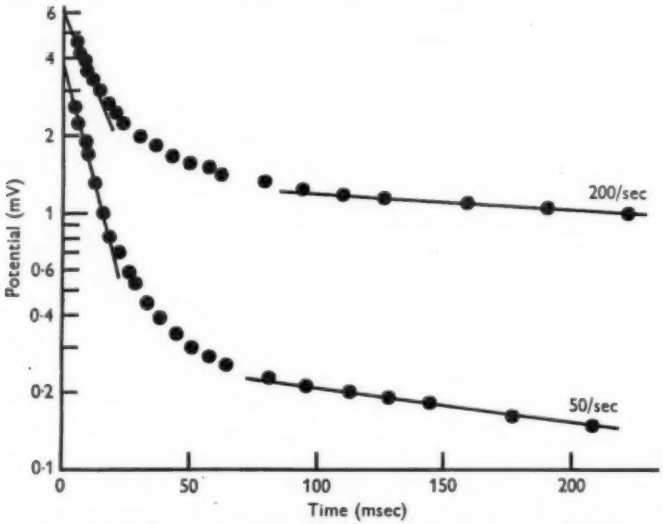

Fig. 4. Time course of the decay of the negative after-potential following a train of impulses at 50 and 200/sec. The axon was bathed in normal Ringer's solution. The ordinate represents the potential on a logarithmic scale, and the abscissa is the time after the end of the last impulse in the train. The straight lines were drawn through the points by eye. Their time constants are as follows: 50/sec, initial, 10·9 msec; 50/sec, terminal, 317 msec; 200/sec, initial, 18·5 msec; 200/sec, terminal, 795 msec. The same axon as in Fig. 3. Temperature 19·5° C.

In the first place, the first three negative after-potentials decayed with approximately the same time constant. There was in some cases a tendency for the time constant to increase when the number of stimuli was increased. This tendency was responsible for the larger values for the time constant of decay of the last impulse in a train as compared with that of the first one, which was described in the previous section.

In the second place, the observed values for the negative after-potentials were in good agreement with the calculated ones. The initial heights of the three negative after-potentials estimated by extrapolation as in Fig. 5 were 0·99, 1·15 and 1·3 mV, respectively. The height of the first negative after-potential remaining at the instant when the second action potential

arose was 0·16 mV, and that of the second one was 0·2 mV. Thus, the calculated values for the initial heights of the second and the third negative after-potentials are $0·16+0·99 = 1·15$ mV, and $0·2+0·99 = 1·19$ mV as against the observed values of 1·15 and 1·30 mV, respectively. The difference is small in view of the errors introduced by the extrapolation procedure.

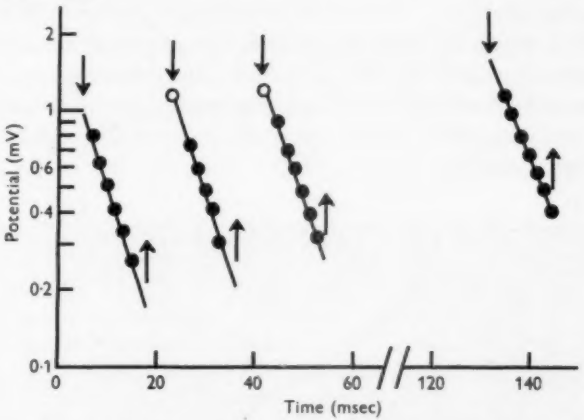

Fig. 5. Time course of the decay of the negative after-potential during a train of impulses of 50/sec. The axon was bathed in normal Ringer's solution. The first, second, third, and eighth impulses are illustrated. The ordinate represents the potential on a logarithmic scale, and the abscissa is the time measured from the beginning of the train. The closed circles are the measured points. The straight lines were drawn by eye. The time constants were: first, 10·7 msec; second, 11·0 msec; third, 12·0 msec; eighth, 13·9 msec. The open circles represent the potential immediately after the end of the second and third spikes, calculated by adding the initial height of the first negative after-potential to the after-potential remaining at the instant when the second and third spikes arise. The upward arrows indicate the start of the spikes, and the downward ones the end of the spikes. Temperature 19° C.

Furthermore, it can be postulated, as with squid giant axons, that the amount by which any impulse in a train declines during a period identical with the stimulus interval must be equal to the contribution of a single impulse, if a steady state has been achieved (Frankenhaeuser & Hodgkin, 1956). This also held true for the cockroach giant axons. For example, at the eighth impulse at a frequency of 50/sec, the calculated value was $1·6-0·4 = 1·2$ mV as against the observed value of 0·99 mV.

Since the dependence of the resting potential upon external potassium concentration has already been demonstrated, the results mentioned above are consistent with the view that the negative after-potential is caused by an accumulation of potassium near the membrane.

Increase in potassium concentration near the membrane. The increase in potassium concentration immediately after the spike was estimated from the height of the negative after-potential extrapolated to the end of the spike, and the curve relating potassium concentration to membrane potential, as in non-myelinated nerve fibres of the rabbit (Greengard & Straub, 1958). The mean value for the increase was 2·0 mM with a range of 0·9–3·0 mM.

Effect of changing the external concentration of potassium. Raising the external concentration of potassium from 3·1 to 10·0 mM caused a decrease in negative after-potential, and removing potassium caused an increase. These effects were most clearly seen when repetitive stimulation was applied (Figs. 1 and 2).

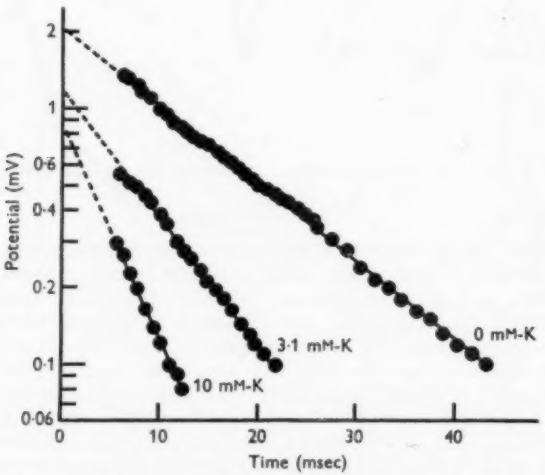

Fig. 6. Time course of the decay of the negative after-potential following a single impulse in varying concentrations of potassium. The ordinate represents the potential (mV) on a logarithmic scale, and the abscissa is the time after the end of the spike. The straight lines were drawn by eye. The time constants are: 10 mM-K, 5·1 msec; 3·1 mM-K, 8·9 msec; 0 mM-K, 15·2 msec. The same axon as in Figs. 1 and 2. Temperature 19·5° C.

Figure 6 shows the decay of the negative after-potential following single impulses recorded from an axon bathed in varying concentrations of potassium. It can be seen that though the decay could be described in each case by an exponential term its time constant was dependent on the potassium concentration, being decreased by raising potassium and increased by lowering potassium. As in normal Ringer's solution, the declining phase of the negative after-potential following a train of impulses in potassium-free solution could not be expressed as a single exponential

term. The initial fast phase decayed with a time constant slightly longer than that of single impulse, and the terminal slow phase decayed with an extremely long time constant. The time constants of both the fast and slow phases were somewhat longer than those estimated in the normal Ringer's solution.

Effect of changing the external concentration of calcium

Calcium-rich solution. The resting potential was increased to some extent by raising the external concentration of calcium from the normal value of 1·8 to 18 mM, the mean hyperpolarization being 3 mV. The magnitude and shape of the action potential underwent some changes, as shown in Fig. 7. In Fig. 8*A* are illustrated the changes of the magnitudes of the

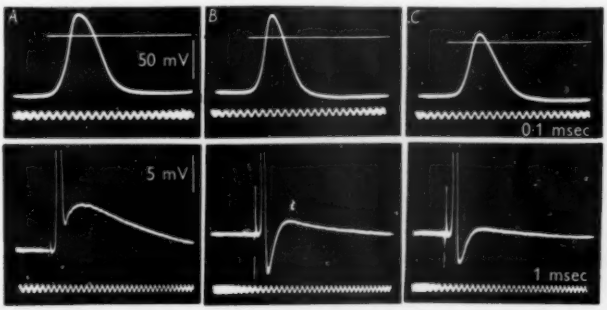

Fig. 7. Records of the action potential showing the effect of changing the external concentration of calcium. The upper records show the spike potential; zero-potential level is indicated by a horizontal line. The lower records show the positive phase and the negative after-potential with higher amplification and slower sweep, the spike potential being too large to be recorded. *A*, in 18·0 mM-Ca; *B*, in 1·8 mM-Ca; *C*, in 0·6 mM-Ca. Temperature 21·5° C.

resting and action potentials. It will be seen that the action potential slightly increased in height and the onset of the falling phase was delayed in the high-calcium solution. Graphical analyses of records of the action potential revealed that the maximum rate of rise decreased by about 20–30 %, while the maximum rate of fall underwent little change.

The post-spike phases were more seriously affected by 18 mM-Ca, the positive phase being depressed or even disappearing, and the negative after-potential being augmented markedly. The declining phase of the negative after-potential could be described by an exponential term with a time constant slightly longer than normal. The negative after-potentials added approximately linearly when repetitive stimulation was applied. The decay following a train of impulses was similar to the normal one in that the initial fast phase finally terminated in an extremely slow phase.

These effects of high-calcium solution were completely reversible on washing with normal Ringer's solution.

Low-calcium solution. Lowering the external concentration of calcium from 1·8 to 0·6 mM caused a depolarization of about 3 mV, and lowering to 0·18 mM caused a depolarization of about 5 mV. Figure 7*C* shows records taken in 0·6 mM-Ca. Changes in the magnitudes of the resting and action potentials after treatment with 0·18 mM-Ca solution are illustrated in Fig. 8*B*. The effects of low-calcium solutions were reversible upon

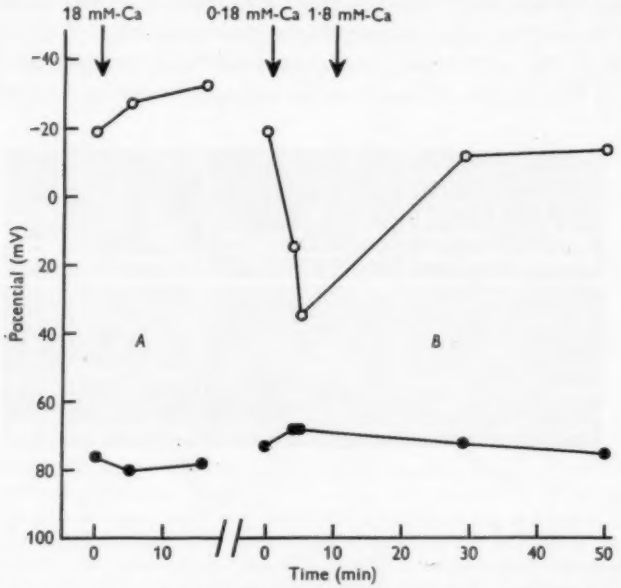

Fig. 8. Effect of changing the calcium concentration on the magnitudes of the resting and action potentials. Two independent experiments on different axons. The ordinate represents the potential; the positive values mean that the outside is positive with respect to the inside. The abscissa is the time in minutes from the beginning of the first measurement made in normal Ringer's solution (1·8 mM-Ca). The closed circles show the resting potential, and the open circles the action potential. The bathing solution was exchanged at the times indicated by the arrows. Temperature, *A*, 19·5° C; *B*, 21·5° C.

washing with Ringer's solution unless the axon was left in the low-calcium solutions for a long period of time. On substituting 0·6 or 0·18 mM-Ca for the normal Ringer's solution containing 1·8 mM-Ca, the action potential rapidly decreased from the initial height of about 100 mV to 50–60 mV. Since the resting potential was only slightly lowered, the peak of the action potential could no longer reach the zero potential level. The maximum rate of rise of the action potential was seriously affected, being lowered to

about 20 % of the initial value. The maximum rate of fall was less affected, being lowered to about 30 %. The positive phase and the negative after-potential were both decreased by the low-calcium solutions. The decay of the negative after-potential following a single impulse could be expressed as an exponential term with a time constant slightly shorter than normal. Both the initial fast phase and the terminal slow phase could also be observed in the decay of the negative after-potential following a train of impulses.

Calcium-free solutions caused a depolarization of more than 10 mV, which was accompanied by a conduction block. These effects were reversible on washing with Ringer's solution shortly after the block.

In no case was spontaneous discharge of the axons observed, or repetitive firing in response to a single shock when the external concentration of calcium was lowered or reduced to zero.

DISCUSSION

The negative after-potential of squid giant axons has been shown to be produced by an accumulation of potassium released during activity (Shanes, 1949*a*, 1951; Frankenhaeuser & Hodgkin, 1956). This explanation also proved to be applicable to the non-myelinated fibres of the rabbit (Greengard & Straub, 1958). The present experiments clearly show that in the giant axons of the cockroach the negative after-potential is also explicable on the same basis.

The exponential nature of the decay of the negative after-potential is well known in many kinds of nerves and muscles. The decay can be described by a single exponential term, for example, in squid giant axons (Shanes, 1949*a*; Frankenhaeuser & Hodgkin, 1956), in non-myelinated nerve fibres of the rabbit (Greengard & Straub, 1958), in crab nerve (Shanes, 1949*b*), and in frog muscle fibres (Frank, 1957). This is also the case in the cockroach giant axons as is shown in the present experiments. As mentioned before, the exponential decline of the negative after-potential of the cockroach axons is indicative of its being passive in nature.

There are two factors which determine the time constant for the decay of the negative after-potential. One is the membrane time constant, which determines the rate of passive decay of the membrane potential when depolarized. The other is the rate at which the released potassium ions diffuse away from the vicinity of the membrane. The time constant for the decay of the negative after-potential would thus be expected to be larger than the membrane time constant. This is actually the case, the former being 9·2 msec as against the value of 4·2 msec for the latter (Yamasaki & Narahashi, 1959*b*).

In the giant axons of the squid the time constant for the decay of the

negative after-potential is estimated as 30–100 msec (Frankenhaeuser & Hodgkin, 1956). This value is very large as compared with the membrane time constant, which is calculated as of the order of 1 msec (Cole & Hodgkin, 1939; Hodgkin, Huxley & Katz, 1949). Therefore it seems reasonable to assume that any external barrier surrounding the axon, through which the released potassium ions diffuse away from the membrane, must be much less effective in restraining the diffusion of potassium in the cockroach axons than it is in the squid giant axons.

Since the nerve preparation is only partly desheathed to make the impalement with micro-electrodes easier, it is likely that the connective tissue sheath surrounding the axons remains intact. The released potassium ions, after diffusing through the immediate barrier already mentioned, have to pass through the connective tissue before reaching the external fluid. It seems likely, therefore, that the terminal slow phase of the decay of the negative after-potential observed after a train of impulses is explicable by the presence of a connective tissue layer which restrains the complete diffusion of potassium. The terminal slow phase seems to be too small in magnitude to be detected when a single stimulus is applied.

The time course of the decay of the negative after-potential has been shown to be independent of the external concentration of potassium in non-myelinated nerve fibres of the rabbit (Greengard & Straub, 1958), in squid giant axons (Shanes, 1949*a*), and in crab nerve (Shanes, 1949*b*). The present results are in contrast to these reports. There is evidence in *Carcinus* axons (Hodgkin, 1947) and in frog muscle fibres (Tamasige, 1951) that the membrane resistance decreases with a rise in the external concentration of potassium. If this also obtains with the cockroach axons, it follows that raising the external potassium concentration causes the membrane time constant to be reduced. Since the rate of decay of the negative after-potential has been shown to be partly governed by the membrane time constant, the reduction of the membrane time constant in potassium-rich solution would undoubtedly cause an acceleration of the decay of the negative after-potential. A slower decay in potassium-free solution would be explained on the same basis.

The negative after-potentials add approximately linearly when the cockroach axon is stimulated repetitively in a calcium-rich solution, and the decay following a train of impulses resembles that of the axon bathed in normal Ringer's solution. These facts may suggest that the negative after-potential is also produced by the accumulation of potassium near the membrane. With respect to the increase in negative after-potential on raising the calcium concentration, however, there is not enough experimental evidence to explain the mechanism involved. There are three possible explanations: (1) a rise in the potassium leakage per impulse,

(2) a decrease in the potassium permeability of the membrane immediately after the spike, and (3) a rise in resting potential. The explanation (1) above is based on the finding in the voltage-clamped squid axon that a rise in calcium causes potassium current to increase at depolarizations above some 40 mV (Shanes, Freygang, Grundfest & Amatniek, 1959). The explanation (2) is based on the evidence in the same axon that at slight depolarizations the rise in potassium conductance is appreciably smaller when external [Ca] is increased (Frankenhaeuser & Hodgkin, 1957; Shanes *et al.* 1959). With such a small rise in potassium conductance, the potential would never get at all close to the equilibrium potential for potassium, thereby elevating the post-spike phase. The explanation (3) is based on the observation on the cockroach giant axon that a hyperpolarization by anodal current causes the negative after-potential to increase (Yamasaki & Narahashi, 1959*b*). The slight rise in resting potential brought about by high calcium would then cause the negative after-potential to increase. However, the available data are inadequate to distinguish between these three possibilities.

SUMMARY

1. The action potential of the cockroach giant axons has been analysed with special reference to the mechanism underlying the production of the negative after-potential.

2. The mean values for the membrane potentials were as follows: resting potential, 77 mV; action potential, 99 mV; positive phase, 4·6 mV; negative after-potential, 1·5 mV.

3. These four values were decreased by raising the external concentration of potassium.

4. During a train of impulses the spike height and the positive phase decreased nearly exponentially, and the negative after-potentials added in a linear manner.

5. The decay of a negative after-potential could be described by a single exponential term with a mean time constant of 9·2 msec. The time constant was decreased by raising the external potassium. The decay following a train of impulses showed in addition a terminal slow phase with a time constant as long as 500 msec or more.

6. Raising the external concentration of calcium caused a slight increase in resting and action potentials, a marked increase in negative after-potential, and a slowing of the maximum rate of rise of the action potential, whereas it had little effect on the maximum rate of fall.

7. In high-calcium solution the decay of the negative after-potential could be expressed as a single exponential term with a time constant slightly longer than that in the normal Ringer's solution.

8. The negative after-potential can be interpreted as being due to the accumulation of the released potassium in the immediate vicinity of the nerve membrane. The increase in potassium concentration was estimated as about 2·0 mM.

We are indebted to Dr S. Hagiwara for criticism during the course of this work. Thanks are also due to Miss S. Hamada for technical assistance.

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THE CHLORIDE CONDUCTANCE OF FROG SKELETAL MUSCLE

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It has long been established that the cell membrane of skeletal muscle is permeable to chloride ions (Boyle & Conway, 1941), but the magnitude of the chloride conductance did not immediately attract attention. Recently Hodgkin & Horowicz (1957*b*, 1959*b*) have shown that under some conditions changes in the chloride concentration ratio may have a greater effect on the membrane potential than equivalent changes in the distribution of potassium; and Hutter & Padsha (1959) have found a decrease in the membrane conductance by more than one half on replacement of chloride by less permeant anions. The indication is that chloride contributes the major share to the membrane conductance.

The aim of the present experiments was to determine the proportion of the resting membrane conductance attributable to chloride by measuring the conductance change on replacement of chloride by an impermeant anion. Sulphate is know to be impermeant (Conway & Moore, 1945), but has the disadvantage of reducing the concentration of ionized calcium. The monovalent anions methylsulphate and pyroglutamate (Boistel & Fatt, 1958) have therefore been used as substitutes for chloride. The results show that in the resting state chloride ions account for 68% of the total membrane conductance. The opportunity has also been taken to study the influence of chloride on the electrical and mechanical response of skeletal muscle.

METHODS

Solutions

The chloride solution contained (mM) NaCl 111, KCl 2·6, $CaCl_2$ 1·8, $NaHCO_3$ 2·2. Methylsulphate solution was made by replacing NaCl by an equivalent amount of $NaCH_3SO_4$ (Hopkin and Williams Ltd). Pyroglutamate solution was made by replacing NaCl with Na-pyroglutamate prepared from L-pyroglutamic acid (L. Light and Co. Ltd) and NaOH, the pH of the final solution being adjusted to 7·4. In some experiments it was convenient to abolish the mechanical response of the muscle and hypertonic solutions were then used (Hodgkin & Horowicz, 1957*a*). These contained (mM) NaCl or $NaCH_3SO_4$ 277, KCl 2·6, $CaCl_2$ 1·8, $NaHCO_3$ 2·2.

* Bayliss–Starling Scholar.

Measurement of membrane potential and resistance

Sartorii of frogs, and occasionally m.ext.dig. IV, were carefully isolated and mounted on a cambered strip of Perspex lying in a small bath (capacity 5 ml.). Solutions of the desired composition were passed continuously over the muscle. In some experiments the reservoirs containing the solutions were kept at 0° C so that the temperature of the preparation could be lowered to varying extents by adjusting the rate of flow of solution. The temperature of the bath was recorded with an electrically insulated copper-constantan thermocouple.

Resting potentials and action potentials were recorded with intracellular micro-electrodes filled with 3 M-KCl. Usually the micro-electrode was connected through an Ag-AgCl half-cell incorporating an agar-Ringer bridge to one grid of a double-sided cathode follower input, while the other grid was connected to a second half-cell in direct contact with the bath fluid. In these circumstances a junction potential of about 8 mV (micro-electrode negative) appeared on replacing chloride ions in the bath fluid by methylsulphate. In later experiments this was avoided by inserting another KCl micro-electrode between the second half-cell and the bath fluid, thus making the voltage recording system symmetrical. To record action potentials in isotonic solutions the fibres were stimulated with a second intracellular micro-electrode. This limited excitation to one fibre and so minimized the danger of the electrode slipping out of the cell. A difficulty in this technique was that low-resistance micro-electrodes had to be used in order to pass sufficient current to excite the fibres, and their insertion was often accompanied by a small fall in the resting potential. In hypertonic solutions, when the mechanical response was absent, we therefore preferred to stimulate through a pair of external electrodes made of fine wire, insulated almost to the tips. To avoid stimulation of nerve fibres the electrodes were placed at the pelvic end of the muscle, or the preparation was curarized with 5×10^{-5} tubocurarine chloride.

Measurements of membrane resistance were made with two intracellular micro-electrodes inserted close together in the same fibre, one to record the membrane potential and the other to pass current through the membrane (Fatt & Katz, 1951). In these circumstances the relation between changes in membrane resistance or conductance and changes in the steady-state electrotonic potential produced by a weak constant current pulse is given by

$$r_m/r'_m = g'_m/g_m = (V/V')^2,$$

where r_m, g_m and V are membrane resistance, membrane conductance and steady state electrotonic potential, respectively, in one solution and r'_m, g'_m and V' are the same quantities in a second solution. In many experiments, current pulses of several different strengths were used and the relation between V and total membrane current, I, was plotted. These curves varied in shape in the different solutions used, in a way which implied that the conductance changes observed were voltage-dependent. In order to facilitate discussion, the results have been presented also as plots of the membrane current density, i, required to produce a uniform change in potential, V, over the whole fibre, found by using the relation $i \propto I\,(\mathrm{d}I/\mathrm{d}V)$ (Cole & Curtis, 1941; Burke & Ginsborg, 1956). Changes in g_m are then directly proportional to changes in the current density producing a given voltage displacement.

To check whether movement of chloride out of the micro-electrode was responsible for some of the features observed, one of the micro-electrodes inserted into the fibre was sometimes filled with 2M-K citrate instead of 3M-KCl. The switching arrangement described by Furshpan & Potter (1958) was then adopted to select either of the two electrodes for passing current.

Measurement of the duration of the active state

Active-state curves were determined by the quick-release method (Ritchie, 1954; Edwards, Ritchie & Wilkie, 1956). The sartorius muscle was held by the pelvic bone while the tendon was connected to a light lever which in turn was linked through a slack wire chain to a mechano-electronic transducer. An electromagnetically controlled stop beneath the lever prevented the muscle from exerting tension on the transducer until a predeter-

mined time after the shock. The muscle was stimulated through a multi-electrode assembly to ensure synchronous excitation of the fibres. All solutions contained 5×10^{-5} tubocurarine chloride and were aerated throughout the experiments with 95% O_2 + 5% CO_2. The experiments were performed at 0° C.

A control experiment on cardiac muscle

Since methylsulphate is a monovalent anion it is unlikely to reduce the concentration of ionized calcium as sulphate is known to do. It seemed desirable, however, to verify this point by assaying the ionized calcium in methylsulphate solution by means of the contraction of the frog's ventricle (McLean & Hastings, 1934; Lüttgau & Niedergerke, 1958). The results from one of these experiments are shown in Fig. 1. The twitch tension was recorded at five different total calcium concentrations, from 0 to 2 mM, successively in chloride, methylsulphate, and chloride solutions. Over the whole range no difference could be observed as a result of replacement of chloride by methylsulphate, confirming that the ionized calcium concentration is not reduced by methylsulphate. On occasion methylsulphate produced a very small increase in tension.

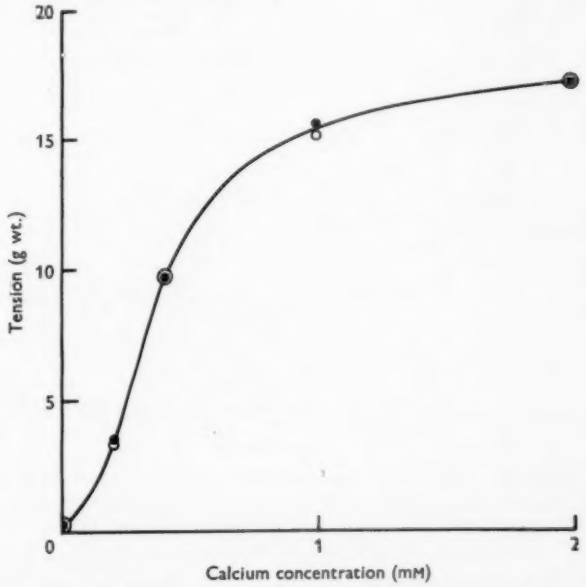

Fig. 1. Variation in the isometric contraction of frog ventricle at different total calcium concentrations in chloride and methylsulphate solution. Abscissa: total calcium concentration (mM). Ordinate: twitch tension in grams. Ventricle perfused with chloride (●) or methylsulphate solution (○).

RESULTS

The impermeability of skeletal muscle to methylsulphate

The determination of the chloride conductance by replacement of chloride with methylsulphate is based on the assumption that methyl-

sulphate cannot penetrate the cell membrane. This is confirmed by the osmotic effect of methylsulphate illustrated in Fig. 2. In this experiment a pair of weighed sartorii was transferred from chloride solution (111 mM-NaCl) to a solution whose principal constituents were 111 mM-$NaCH_3SO_4$ and 111 mM-KCH_3SO_4. As a result their weights decreased by 22%. One of the muscles was then allowed to remain in this solution, to show that potassium methylsulphate, like potassium sulphate (Conway & Moore, 1945), exerts a permanent osmotic pressure due to the impermeability of the membrane to the anion. The other muscle, for the sake of contrast, was transferred to a solution in which chloride replaced methylsulphate. As might be expected, its weight increased again as a result of the uptake of KCl and water.

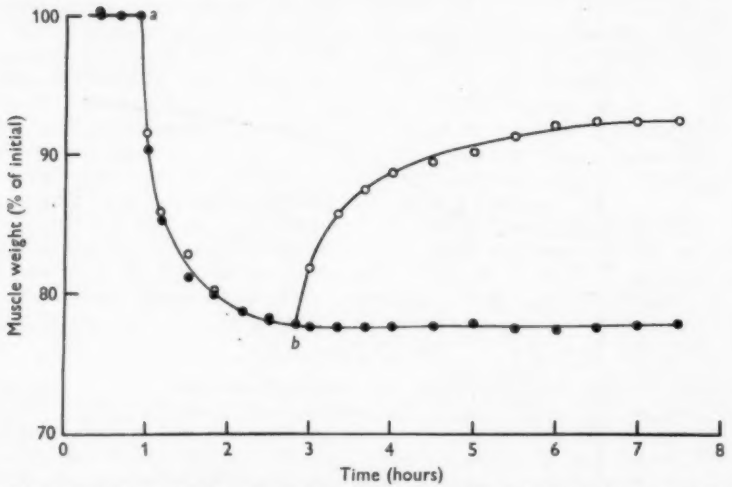

Fig. 2. Effect of K salts of different anions on the weight of frog's sartorii. At *a* the muscles were transferred from chloride-Ringer's solution to a solution whose principal constituents were 111 mM-$NaCH_3SO_4$ and 111 mM-KCH_3SO_4. At *b* one muscle (○) was transferred to a solution containing 111 mM-NaCl and 111 mM-KCl.

The fall in membrane conductance on replacement of chloride by impermeant anion

When chloride in the 'chloride' solution is replaced by methylsulphate, depolarization and twitching of the muscle fibres is usually observed for some time, as a result of reversal of the chloride concentration gradient across the cell membrane (Hodgkin & Horowicz, 1959*b*). To avoid injury we have therefore inserted the micro-electrodes into fibres equilibrated in methylsulphate solution and measured the membrane resistance change

on readmission of chloride. As a rule, hyperpolarizing current pulses of 700 msec duration and known intensities up to about 1×10^{-7} A were used and the electrotonic potential at the end of the current pulse in each solution was measured. In addition the half-decay time was determined in most experiments. Voltage–current curves from two fibres are shown in Fig. 3*A*, *B*. In each case the curve had a steeper slope in methylsulphate than in chloride solution, i.e. the removal of the chloride increased the membrane resistance. As a point of detail, it can be seen that as the membrane was hyperpolarized the slopes of the curves decreased, especially

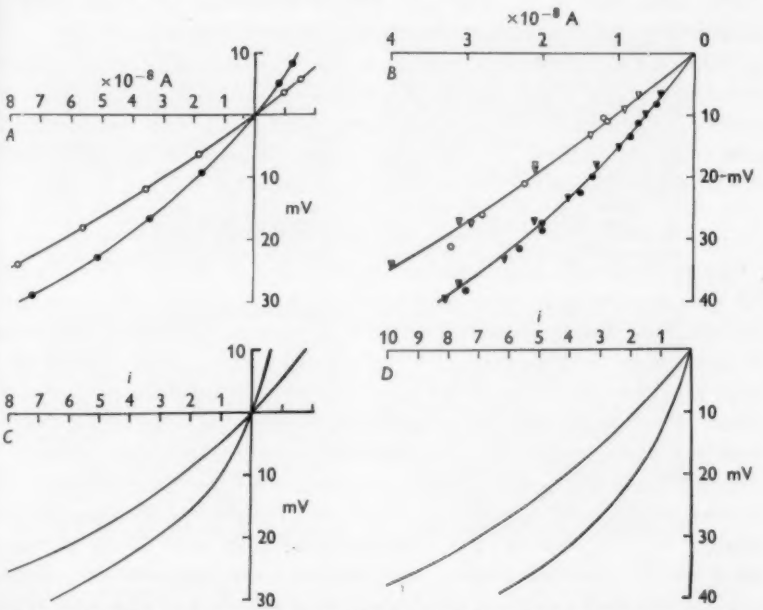

Fig. 3. Effect of replacement of chloride by methylsulphate on the membrane resistance of frog sartorius fibres. *A*, *B*, results from two fibres. Ordinates: steady-state electrotonic potential (V), hyperpolarization downwards. Abscissae: total membrane current (I). ○, ▽ muscle in chloride solution; ●, ▼ muscle in methylsulphate solution. ○, ● current passed through KCl electrodes; ▽, ▼ current passed through K-citrate electrodes. *C*, *D*, are derived from the results in *A* and *B*. Ordinates: as above. Abscissae: membrane current density (i), in arbitrary units calculated from the relation $i \propto I\,(\mathrm{d}I/\mathrm{d}V)$.

in Cl-free solutions; the magnitude of the conductance change therefore varied with the membrane potential. This is brought out more clearly by plotting membrane current density against membrane potential, as the membrane conductance is then given directly by the current density required to produce a given voltage displacement (Cole & Curtis, 1941;

Burke & Ginsborg, 1956). Thus in Fig. 3*C* the ratio of the currents required to produce a hyperpolarization of 5 mV in chloride and in methylsulphate solutions was 1:0·35, while for hyperpolarization of 25 mV the ratio was only 1:0·6. This decline in the effect of methylsulphate was not due to injection of chloride from the micro-electrode (cf. Coombs, Eccles & Fatt, 1955), as it was also seen when electrodes filled with potassium citrate were used for passing the hyperpolarizing currents (Fig. 3*B*, *D*). It therefore indicates that the relative contribution of chloride to the membrane conductance falls as the membrane is hyperpolarized.

The mean fall in g_m near the resting potential, on replacement of chloride by methylsulphate or pyroglutamate, was from 1 to 0·35, $\pm$0·09 S.D. (16 fibres from sartorius and m.ext.dig. IV, mean resting potential 82 $\pm$ 8 mV, 18–25° C). Allowing for the small amount of chloride present in the methylsulphate solution as KCl and $CaCl_2$, this means that total chloride removal would reduce g_m by 68%. In three experiments at 4° C g_m fell to 0·43, 0·32 and 0·33.

The effect of chloride removal on the action potential

The presence of a large chloride conductance in the resting state raises the question as to how far chloride contributes to the repolarization of the action potential in skeletal muscle. The effect was therefore examined of replacement of chloride by methylsulphate on the falling phase of the spike and the after-potential. As we aimed to compare the results with observations on the mechanical response of muscle at 0° C the experiments were made on cooled preparations as well as at room temperature.

A fairly complete experiment on one fibre is illustrated in Fig. 4. The electrode was inserted while the muscle was in hypertonic methylsulphate solution at 4° C and records of action potentials were taken at various times as the solution warmed up towards room temperature. Chloride solution was then run into the bath and after allowing 30 min for equilibration another set of records was obtained. In the illustration the action potentials of the fibre in the two solutions at each temperature have been superimposed. It can be seen that in methylsulphate solution the after-potential was increased both in magnitude and duration at all temperatures and this effect became more obvious in the warm, when the after-potential is prominent even in chloride solution. At 4° C the spike duration also was clearly prolonged, but as the temperature rose and the spike duration decreased this effect grew smaller, until at 17° C no difference between the spikes in chloride and methylsulphate solutions could be detected. The mean prolongation of the spike produced by chloride removal at constant temperatures between 2·8 and 4° C was 25 $\pm$ 7% (measured at 0 mV in 6 fibres in hypertonic chloride and methylsulphate solutions; mean resting

potential in chloride solution 97 ± 7 mV). At room temperatures around 20° C repetitive firing did not usually occur as a result of the increase in the after-potential by methylsulphate, though a second spike was observed to arise on a few occasions when the 'hump' reached about 55 mV (Fig. 5*A*). On hot summer days (28–30° C), however, repetitive firing for over 100 msec was common in methylsulphate and could be recorded with extracellular electrodes (Fig. 5*B*). Replacement of chloride in the chloride solution by nitrate also causes repetitive firing in the warm and a prolongation of the action potential at 0° C.

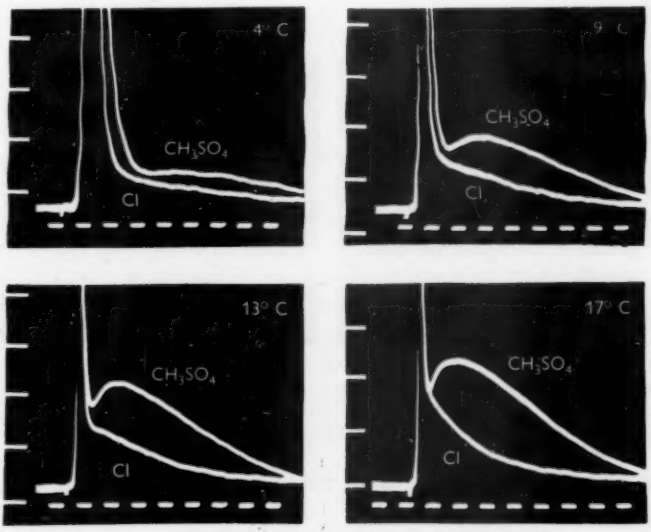

Fig. 4. Effect of replacement of chloride by methylsulphate on the action potential. Records taken from one fibre at different temperatures. Only the lower part of the spike and the after-potential are shown. Muscle bathed in hypertonic solutions throughout. The resting potential at 17° C was 90 mV. Calibrations: 10 mV and 0·01 sec.

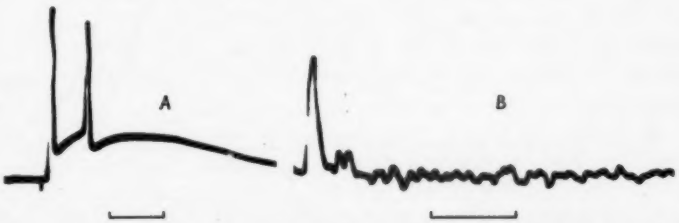

Fig. 5. Repetitive firing in a chloride-free solution. *A*, intracellular record at 20° C. *B*, extracellular record at 28° C. Time calibrations: 20 msec.

The effect of methylsulphate on the duration of the active state of muscular contraction

Several anions which increase the membrane resistance of skeletal muscle, such as Br, NO_3 and I (Hutter & Padsha, 1959), are known to increase the duration of the active state of muscular contraction (Ritchie, 1954; Hill & Macpherson, 1954), and it has been suggested that the two phenomena may be causally linked (Hutter & Padsha, 1959). It seemed interesting, therefore, to test whether the increase in membrane resistance produced by methylsulphate is also accompanied by a prolongation of the active state.

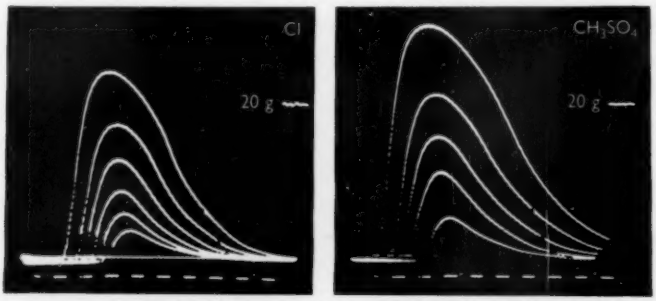

Fig. 6. Effect of chloride removal on the duration of the active state of muscular contraction. Records of tension developed after quick releases at different times from activation in chloride and methylsulphate solutions. Temperature: 0° C. Time calibration: 0·1 sec.

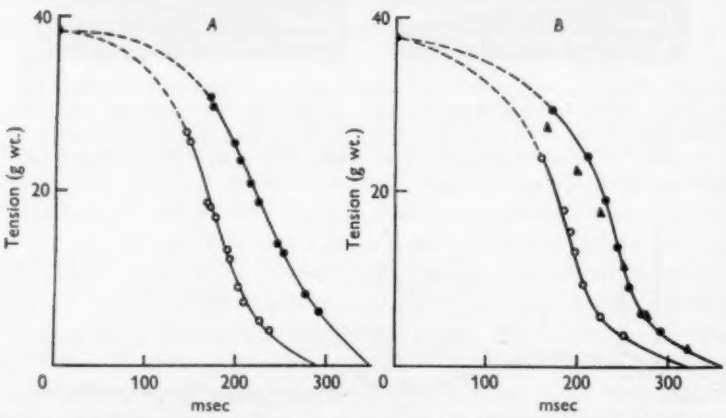

Fig. 7. Time course of decay of active state in chloride (○), methylsulphate (●) and nitrate solution (▲). Results from two muscles. *A* was obtained from the records shown in Fig. 6.

The method used was in all details the same as that described by Ritchie (1954) and Edwards *et al.* (1956). It depends on the argument that at the peak of an isometric twitch, when the contractile elements are not changing in length, the tension is a measure of the intensity of the active state. The maximum tensions developed after quick releases at different time intervals from activation therefore fall on a curve describing the decay of the active state.

Records from one muscle in chloride and methylsulphate solutions are shown in Fig. 6. The top trace in each case is the undelayed isometric twitch. In Fig. 7*A* the curves drawn through the peaks of the tension records in each solution are shown. The moment at which tension first develops in the undelayed twitch has been taken as zero time, and the curves have been drawn as originating from the tetanic tension, since the active state immediately attains its full intensity (Hill, 1949). It can be seen that while the tetanic tension was independent of the anion present, the half-decay time of the active state at 0° C increased from 165 msec in chloride to 220 msec in methylsulphate solution. In an experiment in which the action of nitrate was also tested (Fig. 7*B*) the active state appeared to decay slightly faster in nitrate than in methylsulphate, but more experiments would be needed to confirm such a difference.

At room temperatures near 20° C methylsulphate approximately doubled the twitch tension. At higher temperatures (28–30° C) the response to a single shock in methylsulphate resembled a short tetanus, and records of the electrical activity made with external electrodes showed repetitive firing. Repetitive firing was also found in nitrate under these conditions.

DISCUSSION

The observation that potassium methylsulphate has a permanent osmotic effect justifies the assumption that methylsulphate is a virtually impermeant anion. Provided the membrane current carried by potassium is not dependent on the anion species present, the reduction in the membrane conductance, g_m, on replacement of chloride by methylsulphate should therefore give the contribution of chloride to the membrane conductance, g_{Cl}. The present results show that 68 % of the total g_m may be attributed to chloride; this is in good agreement with the estimate of Hodgkin & Horowicz (1959*b*), who used a different method. The constancy of the fraction of g_m attributable to chloride at different temperatures is consistent with del Castillo & Machne's (1953) finding that the decrease in g_m produced by cooling corresponds to the reduction in ionic mobility in free solution (Robinson & Stokes, 1955).

The fraction of g_m attributable to chloride was found to fall with increasing polarization of the membrane. Further analysis suggests that this is

not due to a fall in chloride permeability, P_{Cl}, but rather the result of an increase in potassium permeability, P_K. Figure 8 is drawn from Fig. 3*C*. The curve in methylsulphate solution is taken as the voltage:current density relation for potassium, the contribution of other cations being neglected. The chloride current density at each voltage was obtained from the difference between the curves in chloride and methylsulphate solutions. According to the equations of the constant-field theory relating ionic current to membrane voltage (Hodgkin & Katz, 1949, equations 2·3 and 2·5, plotted as dotted lines in Fig. 8) an increase in the slope resistance,

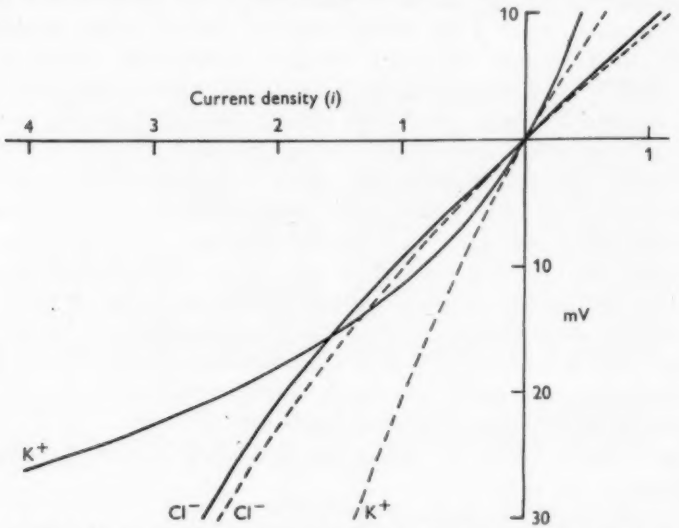

Fig. 8. Relations between membrane potential and ionic current of K^+ and Cl^-. The solid curves are derived from Fig. 3*C* as described in the text. The interrupted curves are relations derived from constant-field theory assuming potassium and chloride to be in equilibrium at the resting potential (84 mV). Hyperpolarization downwards.

dV/di, should be expected as the membrane is hyperpolarized, assuming that the potassium and chloride permeabilities remain constant. This seems to be true for the chloride component, so that there is no evidence for a fall in P_{Cl}. The potassium component, however, shows a progressive fall in slope resistance, as would happen if P_K increased. Katz (1949) has described a rectification in the same direction in muscles in which the chloride contribution was abolished by the use of K_2SO_4; he attributed this effect to the membrane allowing inward K^+ current to pass more easily than outward current. The present results suggest, in agreement with Hodgkin & Horowicz (1959*b*), that this property also exists in fibres

which have not been depolarized. In earlier experiments, when the anion conductance was reduced by nitrate (Hutter & Padsha, 1959) the rectification here described was not so obvious. Presumably the conductance of nitrate itself was not zero and thus masked the effect.

The presence of a large chloride conductance in the resting state suggests that an appreciable inward chloride current may flow during the action potential. That this current plays a significant part in repolarization is shown by the changed time course of the action potential in methylsulphate solution. In the cold both the rapid (spike) and slow (after-potential) phases of repolarization are prolonged. In the warm no prolongation of the spike was observed. Presumably the increase in P_K during the spike (Desmedt, 1953; Hodgkin & Horowicz, 1959*a*) is much greater in the warm than in the cold so that the contribution of chloride to this phase becomes relatively unimportant as the temperature rises. By contrast, the contribution of chloride to repolarization during the after-potential seemed independent of temperature although the magnitude of the after-potential increased greatly at higher temperatures. This finding, and the fact that the change in the half-decay time on chloride removal roughly corresponds to the increase in membrane resistance, are in agreement with the view that the time course of the after-potential is largely determined by the time constant of the resting membrane. Using then the value for the chloride conductance found here, and taking the situation at room temperature when the after-potential is about one-sixth of the spike height, we may conclude that approximately 10–15% of the total repolarizing current flowing during the action potential is attributable to chloride. In this connexion it is interesting to note that Hodgkin & Horowicz (1959*a*) found a deficit between the Na influx and K efflux during the action potential which they attributed to an inflow of chloride, and they refer to Fenn & Cobb (1936) who showed a gain of chloride in mammalian muscle during activity.

The observation that the magnitude of the after-potential varies with the temperature is not in conflict with the view that it largely constitutes a passive decay towards the resting potential, and it need not be supposed that the after-potential is directly of metabolic origin (MacFarlane & Meares, 1958). Benoit, Coraboeuf & Etzensperger (1955) and Desmedt (1953) have shown that the transition between spike and after-potential occurs at a fairly constant membrane potential in a variety of circumstances. Measured from the resting potential, some increase in the amplitude of the after-potential may therefore be expected with a rise in temperature on account of the increase in membrane potential (Nastuk & Hodgkin, 1950). An additional factor, however, may be a much quicker return of excitability in the warm than in the cold, so that a subthreshold regenerative

potential change can arise before the after-potential has decayed too far. This would provide an explanation for the 'hump' on the after-potential and the repetitive firing observed at higher temperatures in methylsulphate solution; for removal of the shunting effect of chloride would facilitate the appearance of a regenerative response.

The effect of methylsulphate on the mechanical response extends the parallelism between an increase in membrane resistance and a prolongation in the active state already found with Br, NO_3 and I (Hill & Macpherson, 1954; Ritchie, 1954; Hutter & Padsha, 1959). On the basis of the comparable electrical records provided here it would appear that at 0° C, when the duration of the active state is best determined, the action potential spike is also prolonged to a similar extent. A close relation between the electrical and mechanical changes may therefore be upheld in this case, although the difference in the time scale of the two events clearly requires that another link must be involved. At other temperatures the situation is not so simple. Around 20° C only an increase in the size and duration of the after-potential can, on this view, be put forward as an explanation for the observed increase in twitch tension. But as the after-potential at this temperature becomes sizeable, it may well suffice to maintain activation for a longer period. Repetitive firing can probably account for much of the greatly prolonged mechanical response seen at still higher temperatures.

SUMMARY

1. The contribution of chloride to the membrane conductance of frog skeletal muscle has been estimated by measuring the fall in conductance on replacing chloride in Ringer's solution by the impermeant monovalent anions methylsulphate or pyroglutamate. The experiments were made with intracellular electrodes for passing current and for voltage recording. It was found that in the resting state chloride ions account for 68 % of the total membrane conductance.

2. The proportion of the membrane conductance due to chloride varied with the membrane potential. The results indicate that this is due to an increase in potassium permeability when the membrane is hyperpolarized, the permeability to chloride remaining virtually unchanged.

3. The effect of chloride removal on the electrical changes during activity has been studied at different temperatures between 3 and 30° C. In the cold the action potential spike is prolonged, indicating that chloride current plays a significant part in repolarization. Around 20° C only the after-potential is increased in size and duration.

4. At 0° C the active state of muscular contraction is prolonged by replacement of chloride by methylsulphate. The twitch tension is approximately doubled at about 20° C in the absence of chloride, and at 28–30° C

the response to a single shock resembles a short tetanus through repetitive firing.

5. The results indicate that changes in the membrane resistance may produce changes in the mechanical response by altering the time course of the electrical events.

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ALTERATION OF SPINAL REFLEXES BY INTERACTION WITH SUPRASEGMENTAL AND DORSAL ROOT ACTIVITY

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With the exception of certain monosynaptic connexions of the large (IA) afferent fibres from the mammalian muscle spindle, it is generally conceded that changes in spinal motoneurone excitability produced by dorsal root fibres are mediated through one or more internuncial neurones. From this it follows that the magnitude of the reflex change produced by given afferent activity is likely to be dependent upon variations of excitability in interneurones which are part of the reflex pathway. The fact that a variety of afferent sources have connexions converging upon some spinal interneurones (Kolmodin & Skoglund, 1958; Hunt & Kuno, 1959) indicates that it is possible for a number of afferent sources to produce alterations in the excitability of an interneurone. That this convergence may play a significant part in the nature of spinal cord output can be deduced if one of several possible functional arrangements proves true. In the case where more than one input produces an excitatory change in an internuncial neurone, it is possible that this cell is part of a pathway common and necessary for a number of segmental and/or suprasegmental motor mechanisms. Thus, as Sherrington (1906) recognized, an interneurone can be an example of the principle of a common pathway to effector organs, though a less general one than the motoneurone. However, the role of interneurones in spinal function may be even more important if impulses arising from peripheral receptors or higher centres were capable of inhibiting interneurones involved in a reflex pathway to such a degree that the pathway would be partially or completely blocked. In the latter situation segmental or centrally initiated movement patterns might be blocked before an effect is produced on the motoneurones, limiting the synaptic play upon the final common path.

Our attention was directed to the role of internuncial neurones in the organization of spinal reflex patterns by a recent report of Eccles & Lundberg (1958). These authors suggested that of the reflex changes in moto-

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neurone excitability evoked by activity in muscle afferent fibres in spinal cats (Lloyd, 1950; Laporte & Lloyd, 1952), only those initiated by IA fibres are present in decerebrate cats. We have been able to confirm, in part, some of the experimental findings of Eccles & Lundberg. It seemed likely that the absence of reflex effect produced by Group II and III muscle afferent volleys in decerebrate preparations could be attributed to an action at some point other than the motoneurone, since the motoneurones involved retained the ability to discharge and to exhibit variations in excitability. From the experiments reported herein it has been concluded that the results reported by Eccles & Lundberg and certain similar changes in reflex reactions are due to interactions at synaptic stations other than the motoneurone.

METHODS

The experiments were performed on cats with intercollicular or high spinal transections. After the brain rostral to the transection was completely destroyed, ether anaesthesia was discontinued and the animals were ordinarily paralysed by intravenous injections of gallamine triethiodide (Flaxedil; Am. Cyanamid), repeated as necessary. A pump with controllable stroke was used to maintain respiration. External heat aided in holding the preparation's rectal temperature between 36 and 38° C.

The lumbar spinal cord was exposed, and ventral roots L6, L7, and S1 were ordinarily cut on one side for recording of reflex discharge. Peripheral muscle or 'cutaneous' nerves from the same side were isolated and divided to be used as afferent sources. Special care in dissection was taken to identify muscles and their supplying nerve branches. Ordinarily all branches of the sciatic and femoral nerves were cut. In a few instances contralateral nerves also served as a source of conditioning input. A volume lead from the dorsal root–spinal cord junction of appropriate lumbar segments was regularly used to monitor the afferent input. Conduction velocities of active fibres in the afferent input were estimated from the latency of components of the dorsal root potentials. *The designations Group I, II and III were applied only to fibres of muscle nerve,* in accord with Lloyd & Chang (1948) and Hunt (1954).

The monosynaptic reflex evoked by a volley in a nerve supplying a muscle or synergistic group of muscles and recorded in a ventral root was usually employed as a test of motoneurone excitability. In several instances ventral roots were left intact and a polysynaptic discharge recorded in a flexor muscle nerve served as the indicator of reflex excitability. The reflex ordinarily was evoked every 5 sec, though recurrence rates of 2 and 10 sec were utilized in some instances. The test monosynaptic discharge was conditioned by afferent volleys in one or two other peripheral nerves. The nerve stimuli were pulses 50–100 μsec in duration, independently controllable in amplitude and timing, and delivered by pairs of Ag wires 0·5–1·5 cm apart. The time–potential area of the reflex discharge (monophasic recordings) evoked by the test stimuli was measured by an integrator which could be 'gated' for the exact duration of the monosynaptic test discharge (Hisey & Perl, 1958). Depending upon reflex variability, 10–25 discharges were measured for each test situation. An identical number of control measurements were alternatively obtained for each interval between conditioning and test stimuli. Comparisons were subsequently made between the alternate controls and tests.

The possible influence of changes in respiration upon spinal reflexes was of particular interest in this study, because of the experiments in which the spinal cord was transected

in decerebrate preparations. The observations reported by Eccles & Lundberg (1958) on change in reflex effect following spinal section in the decerebrate preparation did not indicate whether their animals were spontaneously respiring during part of the time taken in collection of data and then placed upon artificial respiration. It was deemed essential to minimize the effect of respiratory changes upon spinal function as a variable in these experiments. Accordingly, as a routine, the concentration of CO_2 in the expired air was continuously monitored by an infra-red absorption analyser (Liston–Becker). By adjustment of the respiratory pump the CO_2 level could be maintained constant for a given series of observations.

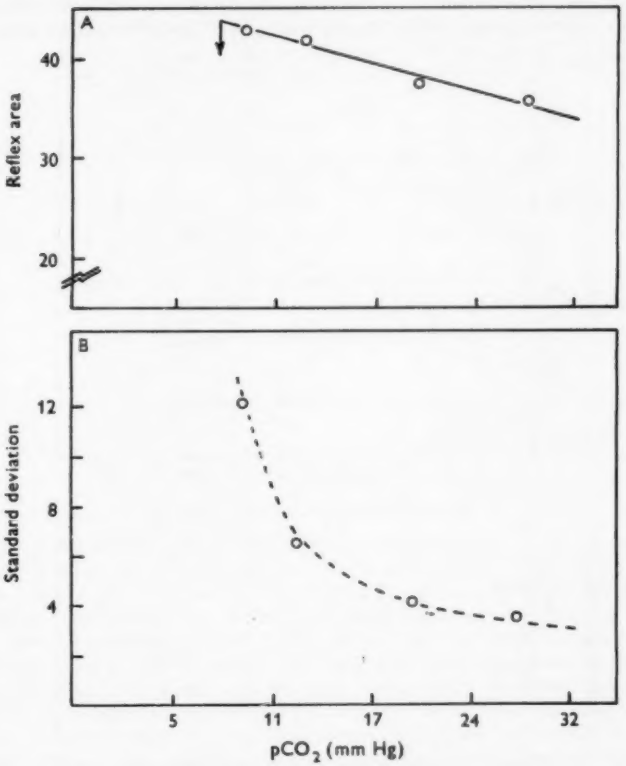

Fig. 1. Effect of overventilation upon the size of the monosynaptic reflex evoked from the posterior biceps and semitendinosus (BST) nerves. CO_2 concentration in expired air was altered by changing tidal volume. Arterial blood appeared bright red in superficial vessels of the spinal cord at all respiratory levels, suggesting full saturation with O_2. The abscissa is scaled in partial pressure of CO_2 (pCO_2). (Salt Lake City mean barometric pressure is approximately 640 mm Hg.) Samples of the expired air were continually drawn from the tracheal tube (at 140 ml./min); the readings plotted were made at the end of expiration. *A*. Ordinate, area under monosynaptic reflex discharge (mean of 40 values for each point) in arbitrary units. *B*. Ordinate, standard deviation of data used to compile *A*. Spinal cat. 3/2/59.

Even limited tests showed that variations in respiratory exchange could produce significant alterations in reflex phenomena. In particular, it was found that the minute volume which maintained CO_2 concentration in the expired air within normal limits (pCO_2 of 35–38 mm Hg) was quite small. The decerebrate preparations when spontaneously respiring, and the artificially respired animal, frequently gave evidence of overventilation by an abnormally low expired CO_2 level. In every instance systematic changes in minute volume indicated that the reflex discharge tended to increase concomitant with a decrease in the concentration of end-tidal CO_2. The increase in the reflex persisted until a very low CO_2 concentration ($pCO_2 = 20$) was reached and then, on occasion, would decrease or fail. Furthermore, with overventilation, as the reflex discharge increased, variation in reflex output also tended to become larger. Typical examples of these changes are illustrated in

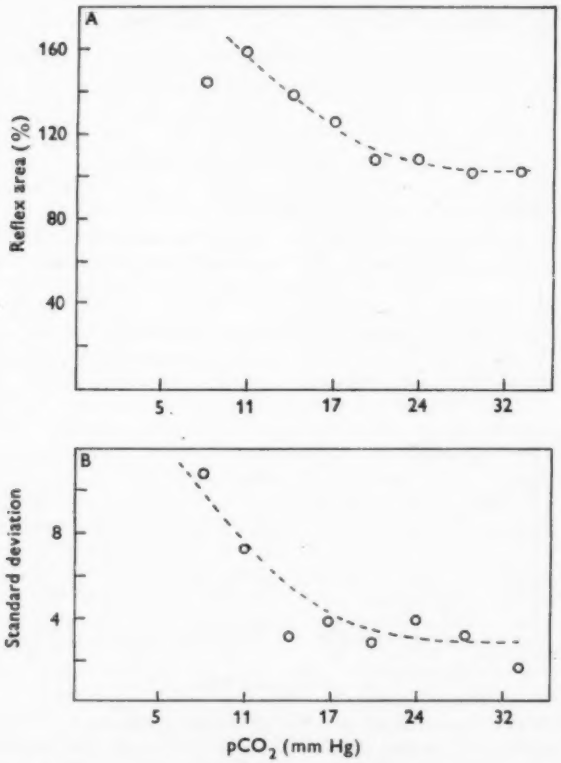

Fig. 2. Effect of overventilation upon the size of a polysynaptic reflex discharge recorded in semitendinosus (ST) nerve. Test reflex evoked from popliteal nerve. General conditions as described in Fig. 1. Continuous sampling of tracheal air at 140 ml./min. End-expiratory readings plotted. Area of first 10 msec of reflex discharge in ST nerve was plotted. *A*. Ordinate, reflex area expressed as percentage of value obtained at pCO_2 of 35 in expired air; average of 20 responses for each point. *B*. Ordinate, standard deviation of individual responses used for *A*. Spinal cat. 23/3/59.

Figs. 1 and 2 for a monosynaptic and polysynaptic reflex discharge respectively. The effects of CO_2 excess on reflex activity were not examined, but have been reported by Brooks & Eccles (1947) and Kirstein (1951).

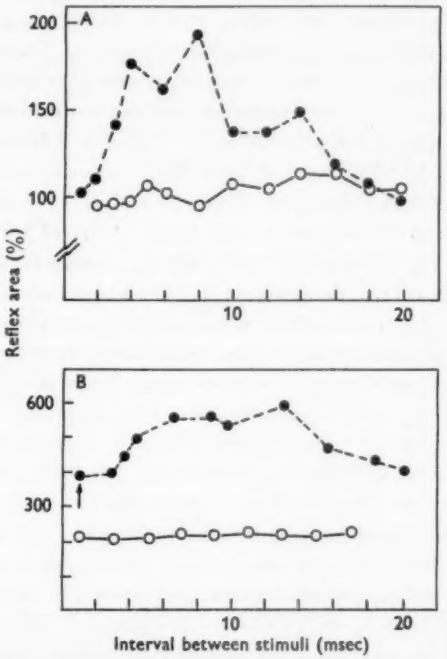

Fig. 3. Conditioning of posterior biceps and semitendinosus (BST) monosynaptic reflex by Group II and III volley of triceps surae origin in decerebrate and in spinal cat. Abscissa in this and subsequent figures gives interval between conditioning and test stimuli. *A*. Ordinate, test reflex area (average of 10) as percentage of alternately obtained control; open circles and solid line show results obtained from decerebrate with intact spinal cord, filled circles and interrupted line give results obtained with identical stimuli following C1 spinal section. Control reflex increased approximately 50% after the spinal transection. 3/2/59. *B*. Ordinate, test reflex area (sum of 15) in arbitrary units: open circles and solid line obtained from decerebrate with intact spinal cord, filled circles and interrupted line give results with identical stimuli obtained after C1 spinal section. Arrow on left indicates direction of change in control reflex size. 1/6/59.

RESULTS

Comparison between decerebrate and spinal preparations

In spinal cats the excitability of motoneurones supplying flexors of the knee and ankle is regularly increased by activity in the Group II (4–12 μ) and Group III (1–4 μ) afferent fibres arising from nerves innervating various hind-limb muscles (Lloyd, 1943, 1950). In a number of decerebrate

preparations, particularly those exhibiting marked extensor rigidity before paralysis, this increase in flexor motoneurone excitability elicited by Group II and III fibres could not be demonstrated by the usual technique of conditioning a monosynaptic reflex evoked from a flexor-muscle nerve. *In our hands, a volley in a substantial number of Group II fibres was accompanied by varying amounts of activity conducted at Group III velocity.* For this reason, in the reflex effects ascribed to Group II below, Group III fibres probably contributed a variable but significant factor. Examples of the lack of Group II effect on the knee flexor monosynaptic discharge in decerebrate animals are shown in Fig. 3*A* and *B* by open circles. When the spinal cord was transected at the upper cervical level and the conditioning curve repeated with stimulation parameters held constant (filled circles), the typical increase in motoneurone excitability appeared. This type of change in reflex effect after spinal transection in a decerebrate animal did not appear in every case; some decerebrate preparations did show the facilitatory Group II effect upon knee flexor motoneurones ordinarily found in spinal preparations. The majority of experiments, however, did give results as illustrated in Fig. 3, thus confirming observations made by Eccles & Lundberg (1958) for this particular conditioning and testing situation. It should be noted that transection of the spinal cord ordinarily increases the size of the knee flexor monosynaptic reflex, as shown in Fig. 3*B*, in which the average reflex area is directly plotted. Increase in flexor reflex size with spinal transection in decerebrate animals was as regular a finding as was the emergence of the Group II effect. Similarly, spinal transection in a decerebrate animal usually resulted in a decrease of a monosynaptic reflex evoked from an extensor nucleus.

In the spinal animal inhibition of extensor motoneurones is part of the Group II-evoked hind-limb reflex (Lloyd, 1950). As Eccles & Lundberg (1958) showed, inhibition of ankle extensor motoneurones by Group II afferent fibres of flexor digitorum longus (FDL) was commonly absent in decerebrate preparations, becoming manifest after spinal transection. Figure 4 consists of results from a decerebrate cat (open circles) showing the lack of change of the triceps surae (TS) monosynaptic reflex response when conditioned by a Group II-containing volley of impulses in the nerve of FDL. Transection of the spinal cord resulted in a 20% decrease in average test reflex amplitude (heavy bars on left of graph) and the appearance of a powerful inhibitory action by the conditioning volley.

In contrast to the above, inhibition of TS monosynaptic reflexes by Group II fibres from posterior biceps and semitendinosus muscles (BST) was regularly present in decerebrate cats, as is illustrated in Fig. 5*A* and *B*, taken from two representative experiments. In the preparation from which Fig. 5*B* was obtained, a Group II volley in TS nerve did not facilitate a

BST reflex until the spinal cord was transected. Thus, while the absence of both an excitatory and inhibitory reflex effect initiated by Group II fibres in decerebrate preparations could be confirmed, a different combination of conditioning volley and test reflex, apparently not tested by Eccles & Lundberg, did demonstrate reflex phenomena from these afferent fibres in the same or similar preparations.

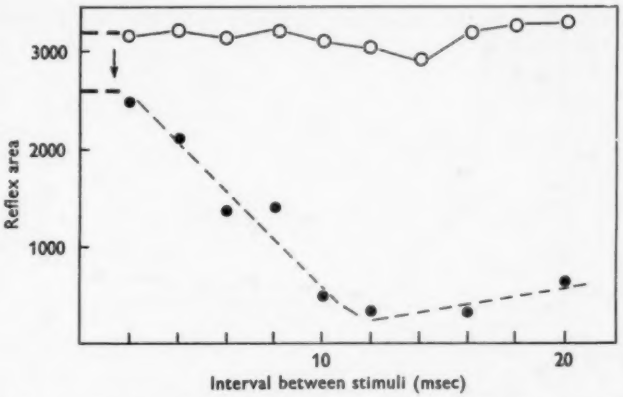

Fig. 4. Conditioning of triceps surae (TS) monosynaptic reflex by Group II and III volley from flexor digitorum longus (FDL) nerve in decerebrate and in spinal cat. Ordinate, test reflex area (sum of 15) plotted in arbitrary units: open circles and solid line show results obtained in decerebrate with intact spinal cord, filled circles and interrupted line give results obtained after C1 spinal section. Heavy bars on left give average control reflex area with arrow showing change following spinal section. 13/4/59.

If the background level of excitability of motoneurones were the only factor determining the presence of Group II reflex effects in decerebrate animals, it would be difficult to explain the following two features of the above results: (1) Spinal transection in some animals resulted in a small change in the monosynaptic reflex coincident with the appearance of powerful Group II and III conditioning actions on the same reflex (Fig. 4). (2) Specific monosynaptic test reflexes showed conditioning effects of Group II and III volleys from some muscle nerves in the decerebrate preparation, while similar volleys in other muscle nerves were ineffectual until the spinal cord was divided.

Group II reflexes are mediated by at least one and probably more internuncial neurones (Lloyd, 1943, 1950; Laporte & Lloyd, 1952). Considering the difficulty of reconciling the absence of Group II reflex effect in the decerebrate animal with changes in motoneurone reflex excitability, it seemed possible that depression of the internuncial

pathway transmitting the Group II effect was responsible for the difference between the spinal and decerebrate animals. It might be supposed that interneurones in the Group II pathway to motoneurones were inhibited by descending impulses to such a degree that they would not discharge when excited by a single volley in Group II fibres from some muscle nerves.

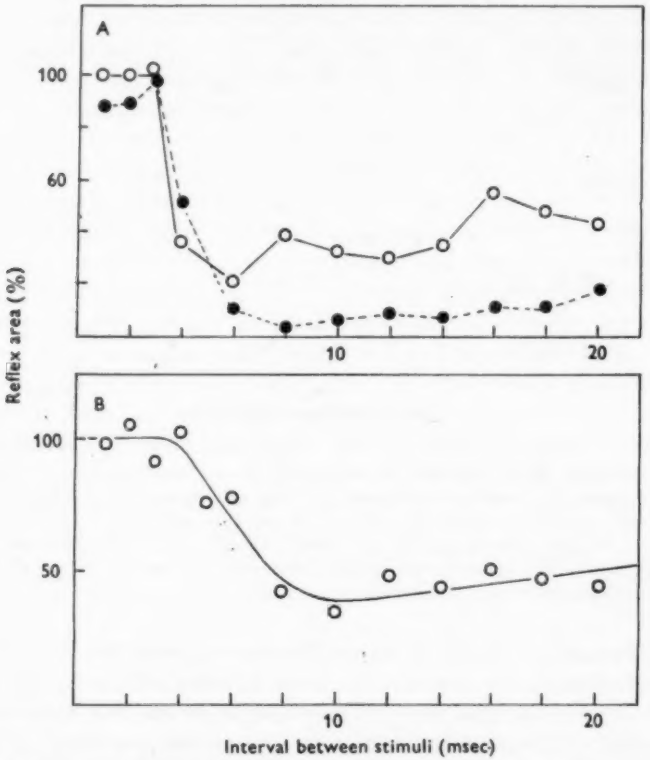

Fig. 5. Conditioning of triceps surae (TS) monosynaptic reflex by a Group II and III volley of knee flexor (BST) origin in decerebrate and spinal cat. *A*. Ordinate, test reflex (average of 10) as percentage of alternately obtained control: open circles and solid line show results obtained in decerebrate before spinal transection, filled circles and interrupted line give results with identical stimuli after C1 spinal section. 29/1/59. *B*. Another experiment; open circles as in *A*. 3/2/59.

Furthermore, it is possible that interneurones mediating the generalized flexor facilitation and extensor inhibition from Group II fibres might also mediate similar reflex effects from another afferent source such as skin. If this were the case, the facilitatory drive to such an interneurone from activity in a cutaneous nerve might counteract some of the depression

produced by suprasegmental structures in decerebrate animals. A test of this line of reasoning is illustrated by the experiment in Fig. 6.

In a decerebrate preparation a Group II volley in the FDL nerve was used to condition a TS monosynaptic reflex. The open circles show that the FDL afferent volley had no effect on the TS motoneurone excitability. The FDL conditioning effect was also tested in the presence of an additional afferent input from the sural nerve. In the latter case, a volley from sural nerve preceded the stimulus evoking the monosynaptic discharge at a

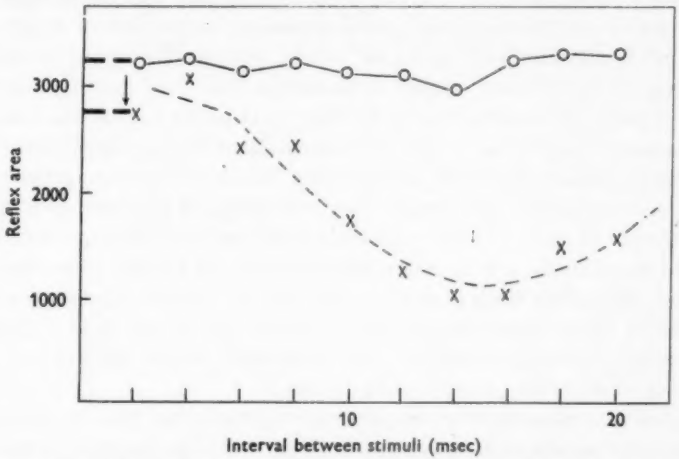

Fig. 6. Conditioning of triceps surae (TS) monosynaptic reflex by a Group II and III volley from flexor digitorum longus (FDL) in decerebrate cat; same experiment as that shown in Fig. 4. Ordinate, reflex area (sum of 15) in arbitrary units: open circles and solid line show test reflex area obtained in decerebrate with intact spinal cord; × and interrupted line give test reflex area obtained with identical stimuli in the presence of an accessory conditioning volley evoked from sural nerve and fixed in time, 7·2 msec before the stimulus eliciting the test reflex. The heavy bars on the left indicate average control reflex area (upper) and the average test reflex area when conditioned by sural alone (lower).

fixed interval of 7·2 msec. At this interval facilitation of flexor motoneurones and inhibition of extensor reflexes could be demonstrated. The heavy bars on the left of Fig. 6 show that the average size of the TS monosynaptic discharge dropped some 15%, owing to the sural input, reflex area being directly plotted in arbitrary units. The FDL volley, in the presence of the background sural effect, produced inhibition of TS motoneurones typical of that found in spinal animals. Again, in the experiment of Fig. 6 the change produced by the sural input in the excitability of motoneurones monosynaptically excited by the TS volley was small compared to the reflex effect which was uncovered. The 'background'

excitability change produced by the sural volley in this experiment is quite similar to that found after spinal transection in the same preparation (Fig. 4).

Effect of accessory conditioning on reflex effect in spinal cat

Another test of the hypothesis that depression of excitability in an interneurone can block a reflex pathway would involve an attempt to reproduce the depression of Group II reflex effects in the spinal cat previously described for the decerebrate animal. The technique used consisted of conditioning the spinal segments important in a Group II reflex with afferent volleys which would initiate polysynaptic activity resulting in spinal cord output opposite to that produced by Group II fibres. The input used to depress the Group II pathway was the relatively long latency effect from cutaneous nerves described by Hagbarth (1952). In spinal animals Hagbarth showed that the short latency facilitation of flexor motoneurones by a sural volley was followed by excitation of some extensor nuclei and inhibition of certain flexor motoneurones. Accordingly, in our experiments, a BST reflex was preceded by a sural volley by about 50 msec. With this timing sural volleys would produce varying degrees of depression of the flexor monosynaptic discharge. A Group II volley from part of TS was used to condition the BST reflex in the absence and in the presence of accessory conditioning by sural.

Figure 7 summarizes an experiment of the type just outlined. The filled circles indicate the effect produced by a Group II volley in the nerve to medial gastrocnemius (MG) upon the BST monosynaptic reflex in a spinal preparation. The usual curve of facilitation appeared with a maximum increase in reflex size of some 900 %. The dotted line to the lower left of the figure indicates the average level of the unconditioned reflex. The BST reflex was then preceded by a near-maximal sural volley (for myelinated fibres) at a fixed interval of 45 msec. The average size of the test reflex was decreased by about 50 %, as is shown by the dash-dot line. When the sural conditioned reflex was employed as a test, the same MG volley as used for the filled-circle curve produced a much smaller excitability change in the pool of BST motoneurones. The method used in setting the timing of the sural volley involved testing the effects on the BST reflex at various conditioning intervals. For the first 5–20 msec such a volley produced powerful facilitation of the BST reflex. During a variable period thereafter, little or no effect was usually found. Between 30 and 50 msec after the sural volley slight-to-moderate inhibition of the BST reflex was evident. This sequence of events precluded subnormality of BST motoneurones discharged during the initial flexor facilitation as a cause of the subsequent depressed BST reflex (Hagbarth, 1952).

A similar experiment on another spinal cat is illustrated in Fig. 8. The solid circles show the effect produced by a lateral gastrocnemius nerve (LG) Group II volley upon a BST monosynaptic reflex. The Group II-evoked excitability change produced in this experiment is much smaller than that illustrated in Fig. 7, though the time course is essentially the same. When the LG conditioning was repeated in the presence of a volley from sural nerve, any change of reflex excitability that appeared was of questionable significance. As can be seen on the left of Fig. 8, the sural volley decreased the BST reflex by some 25 %.

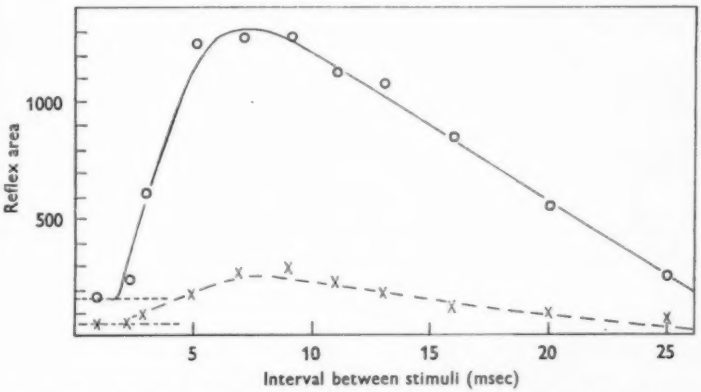

Fig. 7. Conditioning of a knee flexor (BST) monosynaptic reflex by a Group II and III volley from medial gastrocnemius (MG) nerve in spinal cat with and without accessory conditioning by sural nerve. Ordinate, reflex area (sum of 20) in arbitrary units: filled circles and solid line show the test reflex area when conditioned by MG alone, × and interrupted line give the test reflex area in the presence of an additional conditioning volley evoked from sural nerve and fixed in time, 45 msec before the stimulus eliciting the test reflex. The dotted line on the left indicates average control area of the test reflex and the dot-dash line shows the average test reflex area when conditioned by the sural volley alone. 28/5/59.

On the basis of experiments summarized in Figs. 7 and 8, it could be argued that the presence or absence of the Group II effect was a function of the relative excitability of the motoneurones giving rise to the testing reflex, because substantial depression in motoneurone excitability accompanied the depression in reflex effect. On the other hand, it should be recognized that depression of a polysynaptic system producing excitatory effects on flexor motoneurones might be expected to decrease the excitability of these motoneurones to any test. In experiments of the following type, the role of motoneurone excitability in determining the presence of a reflex effect was examined and compared with results obtained with accessory sural conditioning. The procedure used involved changing the

size of the Group I volley evoking the test monosynaptic discharge, thereby altering the size of the test reflex and the excitability of the motoneurone pool from which it was evoked (Hunt, 1955). Figures 9 and 10 were taken from the same spinal preparation and illustrate this type of experiment. In Fig. 9 the open circles show the effects of a LG volley including Group II upon a BST reflex. Reflex area is plotted directly in arbitrary units. No reflex effect was noted for the first 3 msec and points

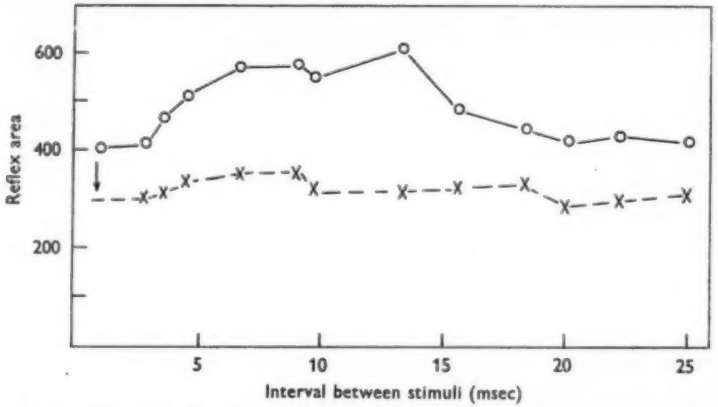

Fig. 8. Conditioning of knee flexor (BST) monosynaptic reflex by a Group II and III volley from lateral gastrocnemius (LG) nerve in spinal cat. Ordinate, reflex area (sum of 10) in arbitrary units: filled circles and solid line show test reflex area when conditioned by LG alone, × and interrupted line show test reflex area in the presence of an additional conditioning volley evoked from sural nerve and fixed in time 45 msec before the stimulus eliciting the test reflex. The initial segment of the solid line indicates the average control area of the test reflex and the initial segment of the interrupted line shows the average test reflex size when conditioned by the sural volley alone. 1/6/59.

plotted during this interval between conditioning volley (LG) and BST volley indicate the size of the control reflex, which stayed quite constant throughout this experiment. The reflex initiated by this BST volley was then preceded by a sural volley at a fixed interval of 54 msec. The conditioning curve by LG was now repeated in the presence of accessory sural conditioning (filled circles) and little or no reflex facilitation appeared. As noted in previous experiments the sural volley decreased the test reflex. The size of the BST volley evoking the monosynaptic test reflex was then reduced so that the average reflex discharge decreased to the size produced by sural conditioning. The conditioning curve was repeated with the identical LG volley and the results are plotted with ×. For these two sizes of BST input the Group II facilitatory effect appears essentially parallel.

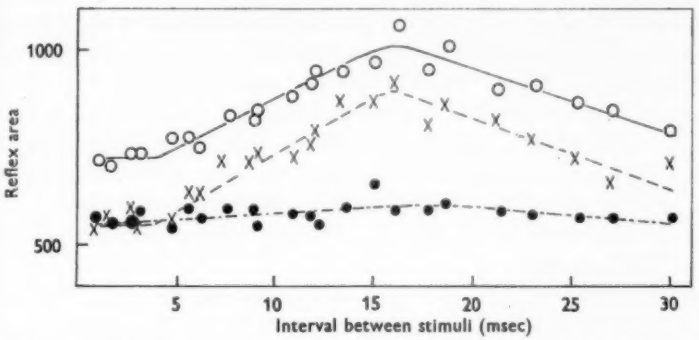

Fig. 9. Conditioning of knee flexor (BST) monosynaptic reflex by a Group II and III volley from lateral gastrocnemius nerve (LG) in spinal cat. Ordinate, test reflex area (sum of 10) in arbitrary units: open circles and solid line show test reflex area when conditioned by LG, × and interrupted line show test reflex area when conditioned by LG for a smaller test reflex (elicited by smaller BST Group I input), filled circles and dot-dash line give test reflex area (elicited by same Group I stimulus used for open-circle plot) when conditioned by LG in the presence of a volley evoked from sural nerve fixed in time 54 msec before the stimulus eliciting the test reflex. 4/6/59.

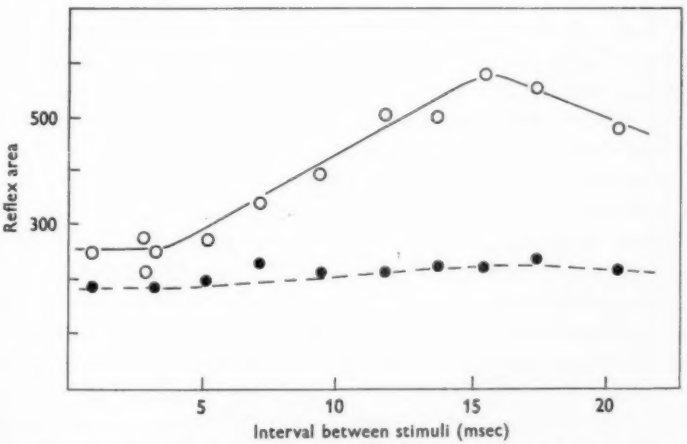

Fig. 10. Conditioning of knee flexor (BST) monosynaptic reflex by a Group II and III volley from lateral gastrocnemius nerve (LG) in spinal cat. Same experiment, conditions, and arbitrary ordinate units as used in Fig. 9 except as noted; note smaller scale of ordinate values. Open circles and solid line show test reflex area, elicited by smaller BST Group I stimulus than was used for observations shown in Fig. 9, when conditioned by LG, filled circles and interrupted line give test reflex area when conditioned by LG in the presence of a volley evoked from sural nerve, fixed in time 54 msec before the stimulus eliciting the test reflex.

Figure 10 is an extension of this experiment. The BST stimulus was further reduced so that the average reflex size was less than one half of smallest average test reflex in Fig. 9. Reflex area was measured in the same arbitrary units as in Fig. 10 (note change in ordinate scale). Conditioning the smaller test reflex with the same LG volley as was used in Fig. 9 produced a curve of facilitation (open circles) parallel to that shown for the larger test reflexes (Fig. 9, open circle and $\times$). This small reflex was now preceded by the sural volley (at 54 msec), additional depression (25 %) of the reflex occurred, and LG conditioning was without effect (Fig. 10, filled circles).

Thus, through a wide range of test reflex size, reflecting large differences in excitability of the BST motoneurone nucleus, the facilitation produced by Group II and III conditioning persisted relatively unchanged. On the other hand, a suitably timed sural volley, altering test reflex size and therefore motoneurone excitability in the same range, could, in effect, abolish the reflexly produced Group II facilitation.

DISCUSSION

The above experiments have been concerned with the effect of activity arising from suprasegmental or segmental sources on Group II and III reflexes. It was shown that in the decerebrate preparation Group II and III fibres from triceps surae nerve and from flexor digitorum longus nerve produced no change in reflex excitability of certain motoneurone nuclei before spinal transection. In contrast, Group II and III fibres arising from the knee flexor muscles were capable of inducing reflex changes in triceps surae motoneurones in the decerebrate preparation. Furthermore, in spinal animals Group II and III reflexes could be drastically depressed or even abolished by prior conditioning of the segments involved with a single volley from sural nerve. It is apparent from these results that some portion of the Group II and III reflex pathway can be interfered with by at least two different kinds of additional activity. Interpretation of the experiments hinges upon determining the locus of the observed interactions. It would be possible for a descending or afferent input to a spinal segment to react with a reflex effect at three places: the primary afferent fibres, internuncial neurones, or the motoneurones. By exclusion we think that the results favour internuncial neurones as the site of the interactions.

Several factors argue against primary afferent fibres as the site of interference with Group II and III reflexes, although interaction between impulses in fibres can occur. It is known that excitability changes can be produced in a group of fibres by impulse traffic in nearby fibres (Katz & Schmitt, 1940; Marazzi & Lorente de Nó, 1944), and that such excitability changes may produce non-specific effects upon a reflex discharge (Laporte

& Lloyd, 1952). Other authors have suggested that impulses in one group of afferent fibres can inhibit or prevent transmission of impulses in another group of fibres, thereby producing rather marked inhibitory effect upon spinal reflexes (Howland, Lettvin, McCulloch, Pitts & Wall, 1955; Frank & Fuortes, 1957). In the study by Howland *et al.* location of reflex inhibition at a presynaptic point was based upon changes in the distribution of current sources and sinks; an analysis which contains unproven assumptions. Frank & Fuortes have described inhibition of monosynaptic excitation which they suggest occurs before the synapse with the motoneurone; however, it should be noted that the interfering volley in their experiments entered the same dorsal root as the volley evoking the test reflex, a situation comparable to that described by Laporte & Lloyd (1952). Activity interfering with reflex effects in the present studies: (1) arose from other than segmental sources as well as dorsal roots; (2) was selective (Figs. 4 and 5); (3) could be overcome by an additional afferent input and (4) was capable of blocking powerful pathways. These factors are in some distinction to inhibition previously ascribed to primary afferent fibres and appear more characteristic of mechanisms acting at a synaptic region.

Elimination of the motoneurone as the point of interaction or block of the Group II reflex effects is based upon the fact that the motoneurones used to test the reflex effect were capable of undergoing the blocked excitability changes, and the assumption that excitability variations among the testing motoneurones was adequately sampled. Support for the first of these points arises from two observations: When triceps surae motoneurones were used as a test of a Group II and III reflex effect in the decerebrate cat, afferent fibres from flexor digitorum longus would not evoke a depression of the testing reflex until spinal section, whereas Group II fibres from the knee flexors evoked a typical inhibitory reflex in the presence of an intact spinal cord. Furthermore, in the spinal animal large changes in the size of a test reflex produced by systematic variation in the testing afferent volley had little or no effect upon the Group II reflex effects, yet a sural volley producing a relatively small change in the test reflex could block the Group II and III reflex (Figs. 9 and 10). In regard to testing and sampling motoneurone excitability, it is assumed that variations in the monosynaptic reflex are a valid measure of the excitability changes in motoneurones supplying a given muscle. This is supported by considerable evidence, which indicates that inputs which are known to produce a particular change in excitability of motoneurones supplying a muscle produce change of similar direction in the size of the monosynaptic reflex discharge evoked from that specific muscle nerve (Lloyd, 1943; Lloyd & McIntyre, 1948; Granit, 1950; Hunt, 1955; Perl, 1957, 1959). An inherent

assumption in the method used for sampling a motor nucleus is that motoneurones which are facilitated or discharged during a monosynaptic reflex are part of a population of motoneurones which are also facilitated or discharged by polysynaptic mechanisms. No published results of the relative excitability of a motoneurone to a monosynaptic and polysynaptic input are available; however, unpublished results (Perl) indicate that motoneurones which can be regularly excited from a muscle nerve monosynaptically can also be easily excited by an input acting through a polysynaptic chain. It seems unlikely that motoneurones of a nucleus supplying a given muscle could be separated into two groups, one of which would not be at least partially tested by a monosynaptic reflex. This follows from the fact that almost all, or all, the motoneurones of a nucleus can be discharged by the monosynaptic reflex under special conditions (Jefferson & Benson, 1953; Henneman, 1954; Hunt, 1955).

Thus, we conclude that the absence of a reflex effect from Group II and III muscle afferent fibres in the decerebrate preparation reported by Eccles & Lundberg (1958), and the inhibition of Group II and III reflex effect by sural volleys described in the present study, result from interactions at synaptic stations other than the motoneurones. The known convergence upon interneurones (see p. 103) makes this thesis a reasonable one. It would appear that interneurones which are part of the Group II and III reflex pathways can be inhibited by activity arising from other sources. In the decerebrate preparation it is presumed that interneurones in a part of this pathway could be depressed by suprasegmental mechanisms to such a degree that activity arising from certain Group II and III fibres would be unable to discharge them, blocking the route to the motoneurone. It also seems probable that those interneurones which are inhibited and cannot be discharged by excitatory activity arising from one Group II and III source (Fig. 4) might be activated by a more powerful input such as might arise from another source (Fig. 5) or from the combination of facilitatory activity arising in Group II and III fibres of a muscle nerve and that produced by the large afferent fibres of a cutaneous nerve (Fig. 6). The same or other internuncial cells in the Group II and III pathway are thought to be inhibited by activity of segmental origin such as that produced by a sural volley which evokes the reflex described by Hagbarth (1952). Again, inhibition of a link in the Group II and III pathway could effectively block the entire pathway unless the facilitatory drive arising from the Group II and III fibres exceeded a certain level. As a final point, it should be mentioned that interruption of a polysynaptic pathway to motoneurones, either excitatory or inhibitory, would produce varying amounts of change in motoneurone excitability depending upon the 'spontaneous' activity of the interneurones.

In our view, the most interesting and significant conclusion which can be drawn from our experiments is that it is possible to limit the segmental influences which play upon motoneurones by interaction, probably at the interneurone level. Thus, one feature of the motor system seems to be dependent upon a type of switching arrangement whereby activity in a given group of fibres or cells can effectively block certain pathways leading to the motoneurone. If one considers all the known segmental and descending pathways which can either excite or inhibit a nucleus of motoneurones, the importance of limiting input to the motoneurone is readily realized. It is quite conceivable that suprasegmental control of motor nuclei in the spinal cord is accomplished by a similar arrangement, thus regulating interference of centrally induced motor acts by local afferent input. In the converse situation, powerful segmental afferent input such as that produced by noxious stimuli might limit the ability of suprasegmental pathways to activate a muscle and thereby limit movement. In any instance involving activity in polysynaptic pathways with conflicting effects upon motoneurone excitability, the net spinal output might vary, depending upon which pathway dominates an interneurone group mediating one or the other effect. It follows that a single electrically evoked volley of impulses in a nerve, in almost every case activating fibres from several types of receptors, might produce opposite effects upon motoneurones as the bias on the internuncial neurones is altered by descending inputs; a not uncommon finding in preparations with parts of the higher nervous system intact.

The organization just suggested can be considered in the light of Sherrington's concept (1906) that sustained spinal reflexes require mechanisms beyond pure summation at the motoneurone. Our present knowledge of synaptic activity in the motoneurone (Eccles, 1957) indicates that this cell does operate primarily by summation. Therefore, some complex and prolonged motor activity known to emanate from spinal mechanisms would not seem possible unless there were some effective means for limiting opposing reflex changes initiated by activation of sensory structures during the beginning phases of motion. Changes in internuncial neurone excitability, as discussed above, would represent a mechanism capable of controlling the number of factors which might alter motoneurone excitability during movement.

Regardless of the site of the interactions which form the subject of this study, it is clear that several spinal and descending systems which are capable of changing motoneurone excitability converge upon common portions of the pathway to the motoneurones. Some descending pathways responsible for some of the features of decerebrate animals exert an inhibitory effect upon part of the pathway which is necessary for the

transmission of Group II and Group III effects from muscle. Furthermore, some of the cutaneous fibres exert a facilitatory effect upon this same portion of the pathway, so that the sum of cutaneous fibre activity and muscle afferent fibre activity can result in transmission through that part of the pathway blocked by the descending influence. Also, those afferent fibres evoking the reflex effects described by Hagbarth (1952), which facilitate motoneurones supplying muscles underlying the skin receptors, exert an inhibitory effect upon part of a pathway involved in transmission of some of the Group II and Group III reflex effects. We suggest that the points of interaction are most probably the interneurones mediating the spinal reflexes evoked from the Group II and Group III afferent fibres of muscle. That interneurones receive such converging input from muscle afferent fibres and cutaneous afferent fibres has been shown (Hunt & Kuno, 1959).

SUMMARY

1. It has been confirmed that certain reflex actions of Group II and III muscle afferent fibres are absent in decerebrate cats and present in spinal cats (Eccles & Lundberg, 1958). Certain other reflex actions of Group II and III fibres were, however, present in the decerebrate preparation.

2. In the acute spinal animal it was possible to mimic the type of reflex depression seen in a decerebrate animal by conditioning with a single volley of impulses from a cutaneous nerve. A sural volley which included small myelinated fibres could partially or completely block the Group II and III facilitation of a flexor nucleus if it arrived some 30–60 msec before the test reflex.

3. The blocking effect produced by descending or dorsal root activity was not dependent upon large changes in motoneurone excitability as determined by a test monosynaptic reflex. Over a wide range, the presence or absence of a given reflex effect evoked from the Group II and III fibres in muscle nerve was not dependent upon the general level of excitability of the motoneurone nucleus from which the test reflex was elicited.

4. It is concluded that the interactions observed between descending activity of the decerebrate preparation and a volley of impulses in the Group II and III fibres of muscle nerve, or that between a volley of impulses in sural nerve and these fibres of muscle nerve, take place at the interneurones involved in the transmission of the reflex effects. The excitability of an interneurone in a pathway is presumed to determine, in part, the presence or absence of polyneuronal reflex effects and perhaps descending functional connexions. The significance of this factor in the control of movement is briefly discussed.

5. Group II and Group III fibres of muscle nerve and certain cutaneous afferent fibres apparently have part of their pathway to the motoneurones

in common, or at least exert common effects upon interneurones essential for reflex changes evoked from the muscle afferent structures.

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Note added in proof

After the present manuscript was submitted, a more detailed report of the study by Eccles & Lundberg appeared [*J. Physiol.* (1959) **147**, 565–584] in which the dissimilarity in reflex response between decerebrate and spinal cat was attributed to the presence of tonic inhibition of spinal interneurones by suprasegmental structures in the decerebrate. This deduction was apparently based largely upon results described by Job [*Pflüg. Arch. ges. Physiol.* (1953), **256**, 406–418]. Job (1953) noted a reversal of certain reflex effects following spinal transection in a decerebrate cat and concluded that, in the intact decerebrate, suppression of interneurones usually mediating a flexor type reflex was responsible for this change. The present study, while dealing with a different experimental situation, supports Job's conclusions that some interneurones in the pathway for generalized flexor reflexes can be sufficiently depressed by supraspinal mechanisms so that block of the reflex occurs.

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THE EFFECT OF 17-METHYLANDROSTENEDIOL ON THE SECRETORY CAPACITY OF THE ADRENAL CORTEX OF RATS

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Treatment with androgens has been shown to prevent, to some extent, the adrenal atrophy which follows hypophysectomy (Cutuly, Cutuly & McCullagh, 1938; Leonard, 1944; Zizine, Simpson & Evans, 1950). For optimal effects treatment has to be started at the time of the hypophysectomy. Adrenal atrophy after 'chemical hypophysectomy', produced by large doses of cortisone or related compounds, can similarly be antagonized by androgens (Winter, Hollings & Stebbins, 1953). The original object of the present paper was the assessment of the secretory capacity of adrenocortical tissue which, though deprived of the normal trophic effects of adrenocorticotrophic hormone (ACTH), partly retains its mass owing to the influence of androgen.

A beneficial effect of androgens on the function of cortical tissue deprived of ACTH seemed strange, in view of the reports of repression of adrenal secretion by androgens under conditions when ACTH was available to the gland (Reifenstein, Forbes, Albright, Donaldson & Carroll, 1945; Brooks & Prunty, 1957). Since, however, the repression of secretion had been observed in man, and the rat might well respond differently, experiments were also carried out on the influence of androgens alone on adrenal secretory capacity of rats.

The term 'secretory capacity' of the rat adrenal refers to a secretion observed under conditions of operative stress, when endogenous release of ACTH causes, at least in animals not given cortisone, near-maximal secretion. Such observations do not give any information about the resting adrenal secretion of a rat subjected only to the 'stress' of the injections.

METHODS

Operative procedures

Adult male Wistar rats, weighing about 250 g, from three different colonies were used. They were housed in groups of four or five per cage in a room kept at 23° C. No attempt was made to train the animals to handling. Usually the rats receiving the same treatment were kept in one cage.

Adrenal vein blood was collected in ice-cooled tubes for 15 min periods under pentobar-

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bitone anaesthesia (45 mg/kg intraperitoneally) as described by Vogt (1955). Heparin (1000 i.u./kg), blood, saline and ACTH were administered via a cannulated femoral vein. The blood pressure was recorded from the femoral artery by means of a mercury manometer. Donor blood was obtained from the carotid arteries of large heparinized rats anaesthetized with ether. It was infused during the collection of adrenal vein blood whenever the blood pressure began to fall.

Drug administration

In the first experiment 1 mg of 17α-methylΔ^5-androstene-3β-17β-diol (MAD) was given to one group, 5 mg cortisone acetate to a second, the two steroids combined to a third, and solvent alone to a fourth group of rats. Injections of a 0·1 % suspension of the steroids in 10 % ethanol were made daily subcutaneously for 9 days.

In the second experiment larger doses were used, the vehicle was oil, and cortisone was replaced by cortisol. The steroids were dissolved in acetone and added to hot arachis oil, and the acetone was evaporated. During evaporation and cooling the steroids crystallized out, so that a mixture of dissolved and suspended material was injected. The doses (each in 0·4 ml. oil) were 2 mg MAD daily for 3 days and 4 mg daily for 6 days, given subcutaneously on their own to group 1 and combined with 5 mg cortisol acetate to group 3. Group 2 had 5 mg cortisol acetate only, and group 4, 0·4 ml. oil.

In the third experiment, done on heavier rats, 5 mg MAD was injected in oily suspension daily for 7 or 8 days.

Chemical procedures

Extraction. The blood was centrifuged, the plasma weighed in tared glass-stoppered test tubes (107 × 18 mm), and stored in the freezer at −17° C until analysed. For analysis the plasma (0·6–2·0 ml.) was thawed, 4–10 μg cortisol added as a check on recoveries of the steroids, and the volume made up to 4·5 ml. with water. The mixture was washed with 5 ml. petroleum ether (b.p. 40–60° C) by inverting (not shaking) the tube 50 times. After centrifugation, the petroleum ether layer was aspirated off and discarded. Five millilitres of an ethylacetate-ether mixture (2:1) was added and the stoppered tubes were shaken vigorously 100 times and centrifuged for 1 min. 4·5 ml. portions of the solvent layers were transferred with an all-glass syringe and a long needle to fresh tubes. These portions were taken to dryness under a stream of air at a temperature not exceeding 50° C. The residues were dissolved in 0·2 ml. methylene chloride and each solution was applied as a 1 cm^2 spot to 2 cm lanes, separated by 1 cm cut-out strips, of Whatman No. 2 filter paper for chromatography. Each sheet of paper had a blank lane and one or two lanes with a mixture of 10 μg each of cortisol and corticosterone.

Chromatography. The method of Bush (1952) was used. The solvents were benzene (500 ml.), methanol (275 ml.) and water (225 ml.) (Bush & Sandberg, 1953), and the temperature was 26° C. The equilibration period was usually during the night and the running time 3·5 hr. The steroids were visualized on the developed chromatogram by an ultraviolet lamp emitting light at 240 mμ.

Elution and colorimetry. Under the guidance of the control lanes, corresponding sections of the plasma and the blank lanes were cut out and eluted with a mixture of ethyl acetate and methanol (2:1) in an all-glass eluter (Saffran & Sharman, 1960). The solvent was evaporated with a stream of air and the residue in each tube was dissolved in 1 ml. of 95 % ethanol. To each tube was then added 0·1 ml. of a 1·25 % solution of tetramethylammonium hydroxide in 95 % ethanol, and 0·1 ml. of a freshly prepared 0·5 % ethanolic solution of blue tetrazolium. After incubation for 1 hr at 23° C, 0·1 ml. of a 10 % solution of acetic acid in 50 % ethanol was added to stop the reaction. The opical density at 520 mμ was determined in the microcells of the Unicam spectrophotometer. The readings followed Beer's Law up to 15 μg of pure steroid; the amounts of steroid in the plasma extracts usually did not exceed 10 μg.

Recoveries. Recoveries of added cortisol averaged 77·5 ± 2·4 %. The elution from the paper was practically complete (Saffran & Sharman, 1960).

RESULTS

Control rats

The left adrenals of untreated rats, or of rats given the vehicles for 9 days, secreted a mean of $26{\cdot}9 \pm 2{\cdot}5$ (S.E.) μg of corticosterone/g adrenal/min (Table 2), a figure which agrees well with those previously found in this laboratory (Vogt, 1955; Holzbauer & Vogt, 1957). Corticosterone was the main component secreted, but occasionally an ultra-violet absorbing spot, which did not reduce blue tetrazolium, with an R_F a little less than corticosterone, appeared on the chromatogram.

Effect of androgens and 17-hydroxy-corticoids

The androgen used was that selected by Winter *et al.* (1953), methylandrostenediol (MAD), and the dosage of steroids in Expt. 1 was, per kilogram body weight, that employed by these authors. Yet, as is shown in Table 1, the adrenal weight was only reduced to 62% of normal by the cortisone,

TABLE 1. Weight of left adrenal of rats (mg; mean ± S.E.M.)

Treatment (9 days)	Expt. 1	Expt. 2
None	$18{\cdot}2 \pm 0{\cdot}6$	$19{\cdot}7 \pm 0{\cdot}6$
MAD	$18{\cdot}0 \pm 0{\cdot}9$	$18{\cdot}7 \pm 1{\cdot}3$
E or F	$11{\cdot}3 \pm 1{\cdot}5$	$8{\cdot}2 \pm 0{\cdot}9$
MAD + E or F	$13{\cdot}3 \pm 1{\cdot}5$	$12{\cdot}2 \pm 0{\cdot}7$

Daily dose per rat in Expt. 1: 1 mg methylandrostenediol (MAD), 5 mg cortisone acetate (E); in Expt. 2: 2–4 mg MAD, 5 mg cortisol acetate (F).

and the repair by the combination of MAD with cortisone was not statistically significant. Differences in strain or the greater age of our rats (Leonard, 1944) may account for these differences. In Expt. 2, therefore, the dose of MAD given was twice, and later four times, that given previously, and cortisone was replaced by the more potent cortisol. In Table 2 the results of all experiments are pooled as the changes in secretion obtained were only somewhat less pronounced with the smaller dosage.

Table 2 (groups 1 and 2) shows that MAD, given on its own, did not change the body weight, adrenal weight or blood flow through the adrenal, but reduced adrenal secretion of cortisone to one half. Cortisone or cortisol, given alone (group 3), also caused, per gram of tissue, a depression of secretion to about one half; owing, however, to the atrophy of the adrenals in this group, the hourly secretion per gland was significantly lower than in group 2, amounting to $9{\cdot}2 \pm 2{\cdot}1$ μg in contrast to $16{\cdot}5 \pm 1{\cdot}6$ μg. Since body weight had also fallen, the secretion figures per gland and per kilogram body weight (Table 2, last columns) were not significantly lower in group 3. The blood flow through the atrophic adrenals

was also reduced, but this cannot account for the fall in secretion, since per gram of tissue the blood flow was larger than normal.

The combination of MAD with corticoids (group 4), though it produced a significant increase in adrenal mass, did not improve the secretion per gram of tissue. There was, in fact, a further, though not significant, fall, so that the glands, while 28 % heavier, secreted only 5 % more corticosterone ($9{\cdot}7 \pm 1{\cdot}3$ μg/hr); per kilogram body weight there was no difference in secretion (see Table 2, last column). It follows that the cellular material which escapes wasting under the influence of MAD either secretes no corticosterone or only very little. Yet histologically the adrenal tissue may look almost normal under these circumstances (Gaunt, Tuthill, Antonchak & Leathem, 1953).

TABLE 2. Effect of methylandrostenediol and 17-hydroxy-corticoids on secretory capacity of the rat adrenal cortex

Group	Treatment	Body wt.* (g)	Plasma collected in 15 min (g)	Weight of left adrenal (mg)	Corticosterone secreted			
					μg/min/g adrenal	% of normal	μg/gland/hr/kg body wt.*	% of normal
1	None	253 ± 6	$1{\cdot}14 \pm 0{\cdot}07$	$19{\cdot}4 \pm 0{\cdot}6$	$26{\cdot}9 \pm 2{\cdot}5$		111 ± 11	
2	MAD	264 ± 5	$1{\cdot}12 \pm 0{\cdot}11$	$18{\cdot}7 \pm 0{\cdot}7$	$13{\cdot}8 \pm 2{\cdot}1$	51	54 ± 7	49
3	E or F	214 ± 4	$0{\cdot}76 \pm 0{\cdot}14$	$9{\cdot}6 \pm 1{\cdot}4$	$14{\cdot}7 \pm 3{\cdot}5$	55	43 ± 9	39
4	MAD+E or F	218 ± 5	$0{\cdot}78 \pm 0{\cdot}04$	$12{\cdot}3 \pm 0{\cdot}6$	$13{\cdot}4 \pm 1{\cdot}9$	50	44 ± 5	40

Abbreviations as in Table 1; doses listed under 'Methods'. All figures are means $\pm$ S.E.M.
* Weight at end of experiment.

Effect of ACTH

It was possible—and earlier experiments (Zizine, 1956) were interpreted in this way—that the androgen inhibited the release of ACTH in operative stress and that this inhibition, and not interference with adrenal enzyme systems, accounted for the poor secretion from glands of animals treated with MAD alone. This possibility was tested by collecting two samples of adrenal vein blood from rats previously injected with MAD (5 mg daily for 7 or 8 days, Table 3); the first sample was collected before, the second after, an intravenous injection of 1 i.u. ACTH. Under similar circumstances normal rats show only little, if any, increase in corticosterone secretion (Holzbauer & Vogt, 1957); this finding has been interpreted as a proof that adrenocortical secretion is already maximally stimulated by the endogenous ACTH released during the severe operative stress. Table 3 shows that, after the injection of ACTH into androgen-treated rats, there also was no rise in the mean yield of corticosterone, though the efficacy of the ACTH was conspicuous by an increase in adrenal blood flow.

TABLE 3. Effect of an intravenous injection of 1 i.u. ACTH on corticosterone secretion by the adrenals of rats treated with methylandrostenediol (5 mg subcutaneously for 7 or 8 days)

Rat no.	Body wt. (g)	Weight of left adrenal (mg)	Corticosterone (μg/min/g adrenal) Before ACTH	After ACTH	Change (%)
1	375	23·6	16·9	17·8	+ 5
2	300	21·4	18·4	11·8	−36
3	350	16·6	19·7	18·5	− 6
4	280	21·6	5·6	7·1	+30
5	290	18·0	15·9	16·3	+ 3
6	317	23·3	6·9	9·8	+42
			Mean 13·9	13·6	

The mean recovery of added cortisol in these experiments was 76%; no corrections for recovery are made in the Table.

Incubations in vitro

Experiments *in vitro* might help to decide whether androgens inhibit the enzyme systems of the adrenal tissue by direct action. The method of Saffran & Schally (1955) was used to assess the formation of corticosterone by incubated tissue pieces. A first experiment, carried out with normal adrenals incubated in the presence of MAD (10^{-4}), showed no inhibition of synthesis of corticosterone, either without or with added ACTH. Experiments on the adrenals of rats pre-treated with MAD for 9 days are in preparation.

DISCUSSION

When rats are subjected to 9 days of treatment with excess corticoids and then to operative stress, their adrenals secrete, per gram of tissue, half the amount of corticosterone produced by normal glands. This finding is easily explained. Corticoids inhibit secretion of ACTH, and this not only leads to adrenal atrophy, but also to a loss of sensitivity of the remaining adrenal tissue to ACTH, a phenomenon first demonstrated in the hypophysectomized rat by Sayers, Sayers, Fry, White & Long (1944). A second effect of the injection of corticoids, a reduction of the ACTH secreted during the operation, may have contributed to the lowering of corticosterone production; it is, however, unlikely that this effect played an important part, since the stress of adrenal vein blood collection in the rat is so severe that the release of ACTH cannot be suppressed (Holzbauer & Vogt, 1958) by means known to inhibit it in less severe operations (Briggs & Munson, 1955). Provided all the stored ACTH is available for release, there is also little doubt that the store of ACTH in the anterior lobes of rats given corticoids for 9 days is ample to cover any needs; serious depletion occurs only after 3–4 weeks (Fortier, 1959).

The amount of corticosterone secreted per gland and per kilogram body weight represents the quantity available to the individual rat during

stress; nine daily injections of corticoids reduce this amount to 39% of normal. This is still a very good secretion compared to that of 1·6% of normal found in rats subjected to the same operative technique 7 days after hypophysectomy (Singer & Stack-Dunne, 1955).

The low secretory capacity of adrenals of rats given methylandrostenediol is in agreement with the clinical observations of a reduced adrenal activity after androgen treatment in man. The effect might be explained by reduced sensitivity to ACTH, by suppression of release of ACTH, or by direct interference with the enzyme systems of the adrenocortical tissue.

Reduced sensitivity to ACTH is rendered unlikely by Zizine's (1956) demonstration that the adrenals of conscious, unstressed rats, injected with androgens for 6 days, responded to ACTH with ascorbic acid depletion in what appeared to be the normal way. This finding, however, can only be accepted as proof that such adrenals are susceptible to stimulation by ACTH if the normal association between fall in ascorbic acid content and secretion of corticosterone is not disrupted by the androgen.

Suppression of maintenance secretion of ACTH would be difficult to reconcile with the fact that the size of the adrenals is normal in androgen-treated rats. Yet there might be inhibition of the increase in ACTH release which normally occurs during stress. Then it should be possible, provided the evidence that sensitivity to ACTH is unimpaired is valid, to stimulate corticosterone secretion by injecting ACTH during the collection of adrenal vein blood. The failure to obtain such stimulation suggests that the operation had already released sufficient ACTH from the anterior lobe to elicit the highest rate of corticosterone formation of which the adrenal was capable. This leaves us with the conclusion that adrenal secretion was probably limited as a result of impairment by the androgen of some enzymic process in the cortical tissue.

From the individual effects of the two types of steroid, the corticoids and the androgens, it was hardly to be expected that adrenal secretion would improve on combining the treatments. The increase in tissue mass, not very conspicuous perhaps because of the age of the rats, was there nevertheless, but secretory capacity per gram of tissue was diminished, or, at best, unchanged, and secretion per gland remained the same. The extra tissue was obviously not contributing to an improvement of function.

SUMMARY

1. Rats injected with cortisone acetate or cortisol acetate (5 mg daily for 9 days) produce 61% less corticosterone than normal rats during severe operative stress; per gram of adrenal tissue the secretory capacity is reduced by 45%.

2. Injections of methylandrostenediol (1–5 mg daily for 7–9 days) reduce the secretory capacity per gram of adrenal tissue by 49%. If the androgen is given together with cortisone or cortisol, there is no improvement in secretion of corticosterone in spite of an increase in adrenal mass.

3. If 1 i.u. ACTH is injected during the collection of adrenal vein blood from androgen-treated rats, the corticosterone production does not rise.

4. Whereas the suppression by corticoids of adrenal secretion is adequately explained by an inhibition of the release of ACTH, the suppression by androgens must be brought about in a different way.

5. Androgens, in spite of the fact that they may help to maintain adrenal size in rats which have been either hypophysectomized or treated with large doses of corticoids, do not have an ACTH-like action on the secretion of such adrenals.

We are grateful to Organon Laboratories Ltd. for generous supplies of cortisone acetate, cortisol acetate, methylandrostenediol and soluble ACTH, obtained through the courtesy of Dr J. Dekanski.

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THE FORMATION OF HISTAMINE IN THE RAT FOETUS

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The foetus in the rat and man, the two species so far investigated, produces histamine at a high rate (Kahlson, Rosengren & Westling, 1958; Kahlson, Rosengren, Westling & White, 1958; Kahlson, Rosengren & White, 1959). This has been shown in the rat by (1) measurement of the urinary excretion of endogenous histamine, (2) study of the rate of urinary excretion of ^{14}C-histamine after injections of ^{14}C-histidine, (3) determination of the rate of histamine formation in foetal tissues *in vitro*, and (4) investigation of the effect of removing the foetus, without removing the placenta, on the excretion of histamine in the pregnant rat. The urinary excretion shows that in the rat the increase in histamine formation occurs at the 15th day of pregnancy and climbs to a peak in the last 1–2 days before term, when a precipitous fall in the rate of formation ensues. It would seem that the foetal formation of histamine during the last third of pregnancy is due to an increase in histidine decarboxylase activity, because the formation of the amine by foetal tissues *in vitro* is inhibited by semicarbazide and hydrazine in concentrations which are known to inhibit this enzyme.

The rat foetus contains little histamine. Even at the peak of histidine decarboxylase activity and urinary histamine excretion (19th–20th day of gestation) the average concentration of histamine in the foetus is less than in the mother. It thus appears that foetal tissues produce the amine at a high rate, but bind histamine only loosely; and, since the histamine is in a state of rapid turnover, the amount found in the tissue is small.

As the significance of the histamine production in the foetus is not known, it seemed desirable to identify the tissue(s) responsible for the high histamine-forming capacity. Further, to understand the discrepancy between histamine content and its rate of formation in the foetus, it appeared essential to study the binding of histamine in foetal tissues. A preliminary communication of these results has been given to the Physiological Society (Kahlson, Rosengren & White, 1959).

METHODS

Measurement of histamine formation in vitro. The method developed by Schayer and his co-workers, and described in detail by Kahlson, Rosengren, Westling & White (1958; see

note, p. 137) was used. A brief outline only will be given here. The tissues were minced and suspended in 0·1 M sodium phosphate buffer of pH 7·4. Where possible 1·5 g of tissue was used but only 0·5–0·6 g of liver in the experiment of Table 1, column 1; the total volume of the suspension was 3 ml. The samples were incubated for 3 hr at 37° C under nitrogen with 40 μg ^{14}C-histidine. The amount of ^{14}C-histamine formed during the incubation was determined by isotope dilution with inert histamine dihydrochloride as carrier. The carrier was added at the end of the incubation and trichloroacetic acid was added to precipitate protein. The sample was filtered and the filtrate was made alkaline and saturated with Na_2SO_4. The filtrate was then extracted with butanol and the butanol fraction was shaken with hydrochloric acid. This procedure was followed by evaporation of the acidic part to dryness and solution of the residue, mainly histamine dihydrochloride, in water to which picric acid in alcoholic solution was then added. The histamine dipicrate crystals formed were isolated and spread on a plate, and their radioactivity was determined. For further purification the histamine, in the form of dihydrochloride, was treated with *p*-iodobenzenesulphonyl chloride and the crystals of pipsyl histamine were isolated and counted in the same way as the histamine dipicrate.

The measurement of radioactivity was made under infinite-thickness conditions in a gas flow-counter. The crystals were deposited on plates of equal size, so that the exposed surface was equal for all samples. The criterion of purity was constant radioactivity after repeated recrystallizations with different charcoal adsorbents; three to six recrystallizations were needed to obtain constant radioactivity. After each recrystallization at least 1000 counts were recorded from each sample.

Extraction of tissues for histamine. Rat foetuses were removed under ether anaesthesia. The tissues required for study (liver, heart, lungs, etc.) were dissected from each member of a litter and pooled. About 30 min elapsed between the death of the foetuses and the beginning of the tissue extraction. Determinations of the histamine content of tissues were made as described by Gustafsson, Kahlson & Rosengren (1957).

RESULTS

Histamine formation in vitro

The results are presented in Table 1 in terms of counts/min/g tissue and signify the amount of ^{14}C-histamine which is formed in 3 hr from ^{14}C-L-histidine; 1 μg ^{14}C-histamine gives 600 counts/min. The results for the organs were obtained by pooling the tissues in each litter. Three litters were examined on the 19th, 20th and 21st days of gestation, respectively. Histamine excretion by the mother is at a peak on the 19th or 20th day of gestation, and tends to fall on the 21st day.

It will be seen that the foetal liver produces histamine at an enormously high rate. The histamine-forming capacity per gram of tissue is about 100 times higher in the liver than in the rest of the body. It is far greater than in the stomach, which is the most potent manufacturer of histamine in the adult; the foetal stomach itself is much more active in forming histamine than that of the adult. In fact, all foetal tissues investigated are potent producers of histamine. At the 19th day of pregnancy, when the figures are very high, organs small in mass could not be examined because of the limitations of the method used. From the available figures it can be

calculated that the liver accounts for about 80 % of the histamine formed by the foetus. However, it should be emphasized that all foetal tissues examined produce histamine at a rate which is very high when compared with the infantile and adult animal.

On the day before term, when the maternal excretion of histamine is on the wane, the activity of the foetal liver has fallen to the low level found in the new-born, and within 2–3 days the histidine decarboxylase activity of the liver regresses from an unparalleled height to the low level found in the new-born and adult animal.

TABLE 1. Rate of histamine formation in 3 hr in rat foetuses, new-born, and adults, expressed as counts/min/g tissue. 1 μg ^{14}C-histamine gives 600 counts/min

	Foetus 19 days	Foetus 20 days	Foetus 21 days	New-born < 3 hr	Young 2–8 days	Adult pregnant
Whole body	—	3,440	340	860	15	—
Liver	14,000 (23,000)*	8,730	2,280	2,240	5–40	5
Stomach	2,800	—	—	—	—	370–980†
Lungs	1,900	930	150	60	5	90–150†
Heart	—	—	140	30	0	—
Kidneys	—	—	190	—	0	< 5†
Stomach + intestines	—	1,250	150	—	—	—
Brain	10	5	20	5	0	—
Rest of the body (muscle, skin, bone)	130 (220)*	360	70	60	30	—

* Figures in brackets are the means of determinations in three litters; the other figures in this column are from a single litter. All the results in the second column are from one litter, and so also those in the third and fourth.

† Figures quoted from Kahlson, Rosengren, Westling & White, 1958.

Histamine content of foetal tissues

Since the published figures for the histamine content of the foetus are somewhat inconsistent, and because our rats were fed on a special histamine-free diet, it was considered necessary to study the histamine content of the foetus anew. In the developing rat embryo histamine is present at the 9th day of gestation in measurable amount. Subsequently the content of the whole foetus increases progressively, slowly at first and more rapidly after the 12th day (Misrahy, 1946). Dixon (1959) was able to detect histamine only at the 11th day of gestation, a time when Misrahy found 1·3 μg/g in the whole foetus, but confirmed the subsequent continuous increase in histamine content. According to Misrahy the foetus at the 20th day contains histamine in a concentration of 4·6 μg/g, whilst Dixon found less than half this amount; at the 19th–21st day we found 1·3–2·1 μg/g (Table 2) and at the 19th–20th day about 3–5 μg/g (Kahlson, Rosengren, Westling & White, 1958). Greater discrepancies exist in the figures reported for the histamine content of various rat foetal organs. Parratt & West (1957) found a striking increase in the histamine

concentration of the lung (25 times) and of the liver (12 times) at birth as compared with the pre-natal figures and a return to near foetal levels by about the sixth day of extra-uterine life. On the other hand, Dixon noted in the lung a low and constant level during intra-uterine life, climbing rapidly after birth to reach a maximum about the third hour post partum. According to this author the ante-partum levels in the liver vary, the content at birth being high and maintained for the first few hours after birth and then falling precipitously to a low level which does not change after the second day. Misrahy found the content to be the same in the lungs of new-born and adult individuals, whereas the corresponding ratio in the livers was about 4:1.

TABLE 2. Histamine content of tissues of rat foetuses and new-born young. The figures denote μg histamine/g tissue

	Whole foetus	Foetus without liver	Liver	Lung
Foetus, 19th day of pregnancy	—*	2·3	18·6	—
Foetus, 19th day of pregnancy	2·1	1·4	23·5	0·7
Foetus, 21st day of pregnancy	1·3	1·3	4·6	0·7
Young, 6 hr after birth	—	8·5	1·0	1·0
Young, 8 hr after birth	—	10·1	0·9	0·8
Young, 8 days after birth	—	11·7	0·8	—
Adult females (Gustafsson *et al.* (1957)	5·1–6·9	—	—	3·3–8·2

* Not examined.

We have therefore examined two litters on the 19th day of pregnancy, one on the 21st day and one litter at 6 hr, one at 8 hr and one 8 days after birth, pooling the livers, lungs, etc., from the members of each litter. As recorded previously, at the 19th day the histamine-forming capacity of the foetus is about maximal, whilst the 21st is the day of steep regression in the rate of histamine formation. The results obtained for histamine content are shown in Table 2. At the time of maximum histamine production in the whole foetus, although the rate of production *in vitro* is about 10 μg/g tissue/hr, the average total concentration in the body is not greater than a few micrograms per gram of tissue. It is evident that the newly formed histamine is not bound in the foetus but is transferred rapidly to the mother.

Binding of newly formed histamine

To see what proportion of newly formed histamine remains bound, in the sense that it cannot be easily washed away, the following experiments were done *in vitro* on whole foetuses, liver, lung and skin. The minced tissue was incubated with ^{14}C-histidine as already described. After 3 hr incubation the mixture was centrifuged at 1000 *g* for 10 min. The supernatant fluid was decanted and the residue was washed with about 6 ml. of buffer solution

and centrifuged twice. The ^{14}C-histamine content of the tissue mince and the combined supernatants were determined separately. The results are presented in Table 3, which shows that in the whole foetus about 12 % of the newly formed histamine is bound, in the liver 20 %, in the lung 40 % and in the skin 45 %. It should be recalled that of the total formation of histamine in the foetus about 80 % takes place in the liver.

TABLE 3. Binding of newly formed histamine in rat foetuses and in foetal tissues expressed as counts/min/g tissue. A high ratio in the last column indicates a low histamine-binding capacity and vice versa

Tissue	Day of pregnancy	Supernatant (*a*)	Tissue (*b*)	Ratio (*a*)/(*b*)
Foetus (3) } * same litter	17	2100	350	6·0
Foetus (4) }	17	2100	310	6·8
Foetus (2) } same litter	17	2000	220	9·1
Foetus (2) }	17	2200	270	8·1
Foetal liver	20	3490	780	4·5
Foetal lungs	20	210	150	1·4
Foetal skin	20	140	120	1·2

* The figures in brackets denote the number of foetuses pooled in one sample.

These experiments show that the histamine formed in foetal tissues easily diffuses into the surrounding fluid and that only a small proportion of it is retained in the tissue. By contrast, in a similar type of experiment on the skin of the adult female rat, only about 1/3 of the formed histamine was found in the supernatant whilst 2/3 was retained in the tissue (Schayer, Davis & Smiley, 1955).

DISCUSSION

Foetal histamine is predominantly of non-mast-cell origin. In the rat foetus mast cells have been found only in the skin (for references, see Dixon, 1959); yet the histamine-forming capacity of the skin is very low. On the other hand, the ability of non-mast-cell tissues to form histamine in the foetus is unequalled. In the adult the most potent manufacturers of histamine are the stomach wall and the mast-cell tumour; yet a mast-cell tumour of a dog investigated in our laboratory (Lindell, Rorsman & Westling, 1959) was about 100 times less active per unit weight in forming histamine than the foetal liver.

The histamine-forming cells and the intracellular location of histidine decarboxylase remain to be identified. It is considered that the histamine-forming capacity of foetal tissues depends on the activity of this enzyme, since the formation of histamine is abolished by inhibitors of histidine decarboxylase (Kahlson, Rosengren, Westling & White, 1958; Kahlson & Rosengren, 1959*a*). These inhibitors are rather unspecific and do not furnish precise evidence, but the findings agree with the assumption that we are dealing with histidine decarboxylase.

Foetal tissues, in general, do not contain much histamine and the rather

high values in the liver are exceptional, but the rate of histamine formation in this tissue is outstandingly high. Histamine is likely to be formed in the liver in considerable amounts during the dissection and preparation of the samples for extraction and the estimates may not represent the actual level of histamine content at the moment of death. The same holds true for other foetal tissues where the rate of histamine formation is high. These circumstances may in part account for the discrepancies in the figures for histamine content in foetal tissues which have been reported by various authors.

In adult tissues rich in mast cells histamine is not easily extracted and rather drastic procedures, which destroy cell structures, are required. In foetal tissues the situation is entirely different. The histamine, so abundantly formed, diffuses freely into the surrounding fluid; the differences in histamine-binding capacity are shown in Table 3. Only in the skin are mast cells present, and this may explain the position which skin occupies at the upper extreme in binding capacity. From the present observations a generalization can be made concerning non-mast-cell histamine: the amount of histamine yielded on extracting a tissue bears no relationship to its histamine-forming capacity. Similar reflexions have occurred to Schayer *et al.* (1955).

Foetal tissues endowed with a high histamine-forming capacity and a low binding capacity are subjected to histamine in a state of rapid turnover. Little is known about the physiological meaning of this process and whether it operates for the benefit of the foetus, or of the mother or of both. Fortunately it has now become possible to depress histamine formation in experiments on the foetus. Under the combined influence of pyridoxine deficiency and rather small doses of semicarbazide, a carbonyl reacting agent which inhibits a variety of enzymes, including histidine decarboxylase, the histamine-forming capacity can be lowered to about 20 % of normal. As a consequence foetal growth is promptly arrested, the foetus dies and becomes mummified. In controls fed on the pyridoxine-deficient diet for the same period of time, or simply injected with semicarbazide, no abnormalities in the course of pregnancy and foetal development were noted (Kahlson & Rosengren, 1959*a*, *b*). These authors (1959*b*) also found that rat liver tissue regenerating after partial hepatectomy displays a substantially elevated histamine-forming capacity. Further, the histamine-forming capacity is also considerably raised in the tissues of healing skin wounds (G. Kahlson, K. Nilsson, E. Rosengren & B. Zederfeldt, unpublished). Thus, histamine-forming capacity has been found to be associated with three types of growth: foetal, regenerative and reparative. The phenomenon would therefore seem to be related in some way to the general process of growth.

The physiological meaning of the histamine-forming capacity and the consequences of reducing or abolishing it will be further elucidated when inhibitors of histidine decarboxylase which are more specific than semicarbazide become available. The discovery of rich sources of mammalian histidine decarboxylase, such as foetal tissues and gastric mucosa, should be helpful in the search for such inhibitors.

SUMMARY

1. The histamine-forming capacity, the histamine content and the histamine-binding capacity have been determined in a variety of tissues from rat foetuses at various stages of gestation.

2. The histamine-forming capacity is very high until about one day before term and is particularly great in the liver, where it ascends to levels greater than any previously recorded.

3. The histamine content of foetal tissues is below that of the mother in most of the tissues investigated.

4. The histamine-binding capacity of foetal tissues is low: newly formed histamine diffuses freely into the surrounding fluid.

5. As a result of a high histamine-forming capacity and a low histamine-binding capacity foetal tissues are exposed to histamine in a state of rapid turnover.

6. In tissues in which the histamine is of non-mast-cell origin the content bears no relation to the histamine-forming capacity.

7. The physiological significance of the histamine-forming capacity is discussed.

We wish to correct a misprint which appeared in the earlier paper by Kahlson, Rosengren, Westling & White (1958): on p. 338, line 23 for ^{14}C-histidine read ^{14}C-histamine.

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THE PRODUCTION OF RUMEN AND RETICULUM CONTRACTIONS IN DECEREBRATE PREPARATIONS OF SHEEP AND GOATS

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In decerebrate preparations of ruminants contractions of the reticulum similar to those described in conscious animals have been evoked reflexly by changes in conditions in the stomach, including stretch of the reticulum itself and the introduction of acid into the abomasum (Titchen, 1958*a*). A contraction of the rumen usually follows each contraction of the reticulum in conscious animals; in addition rumen contractions occur independently of those of the reticulum (Phillipson, 1939). In decerebrate preparations contractions of the rumen associated with those of the reticulum have been described following stimulation in an afferent sense of branches of the vagus nerves (Iggo, 1956). Rumen contractions were not regularly observed in experiments in decerebrate preparations in which reticulum contractions were evoked by changes produced in it or other parts of the stomach (Titchen, 1958*a*).

These observations in conscious and decerebrate animals suggested that stimuli other than those previously described might have to be delivered for rumen contractions to be evoked consistently together with reticulum contractions in decerebrate preparations. Conditions under which contractions of both the reticulum and rumen had been observed in decerebrate preparations are described below and evidence on the nature of one particular stimulus presented.

METHODS

The animals, methods of their preparation, anaesthesia and decerebration were similar to those described previously (Titchen, 1958*a*). Contractions of the rumen, and particularly those of its dorsal sac, were recorded with frontal-writing levers attached by fine cotton threads led via pulleys and tied to appropriate regions of the rumen exposed by abdominal incisions. This method of recording the activity of the rumen involved less interference with this organ than did the balloon manometer method of recording.

In a number of preparations the spinal cord was transected by crushing; in some others the thoracic and lumbar regions of the spinal cord were destroyed by pithing with a wooden rod inserted into the vertebral canal. In these preparations the blood pressure was maintained by intravenous infusion of solutions of polyvinyl pyrrolidone (Light) or Dextran (May and Baker).

Contractions of the reticulum, either alone or with those of the reticulo-rumenal fold and contiguous structures of the rumen, were recorded from a balloon attached to a water manometer arranged as previously described (Titchen, 1958*a*). By changing the shape of the balloon, stretch could be applied either to the reticulum alone, or simultaneously to both the reticulum and reticulo-rumenal fold. This was done by means of a string tied to the apex inside an ovoid rubber balloon and secured between the polythene and glass tubing which connected the balloon to the manometer. Thus if the string were kept taut the balloon, on distension, was spherical, but it elongated on distension if the string were left loose. The spherical balloon, if carefully placed, when distended stretched the reticulum alone; on elongation, and distended with the same volume of water, the balloon stretched both the reticulum and the reticulo-rumenal fold or orifice, through which it projected. The relations of the reticulum and the reticulo-rumenal fold may be seen in Fig. 1 drawn from a photograph of a paramedian section of a sheep's trunk.

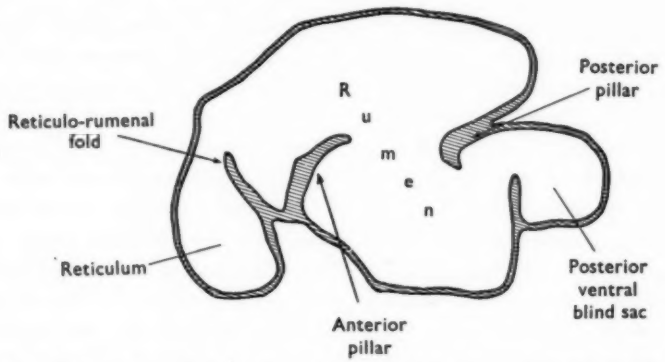

Fig. 1. Outline drawing (from a photograph) of a paramedian section of the reticulum and rumen of a sheep showing the position of the reticulo-rumenal fold.

The position of the balloon could be judged by insertion of a probe along the tube to which the balloon was attached. With experience it proved possible to estimate where the apex of the balloon lay by determining the direction which the probe took and the length which could be inserted without meeting any great resistance. In some experiments this estimate of the position of the balloon was confirmed by direct observation through fistulae previously prepared in the partially exteriorized reticulum (Titchen, 1958*b*) or in the anterior part of the dorsal sac of the rumen. In many others, confirmation of its position was obtained by deeply anaesthetizing the preparations and then, by direct inspection, determining the position of the balloon with various degrees of distension and with the string tied to its apex left loose or kept taut.

RESULTS

The experiments described were concerned primarily with the responses of the reticulum and rumen to two forms of stimulation, namely, stretch of the reticulum alone and stretch of the reticulo-rumenal fold combined with stretch of the reticulum. The effects of some other stimuli previously employed in an examination of the reflex responses of the reticulum

(Titchen, 1958*a*) were compared with these. The observations were undertaken in decerebrate preparations of sheep and goats over periods up to 17 hr after transection of the brain stem and after the administration of ether had ceased. The effects of the stimuli described were, as a rule, examined after periods of 10–120 min quiescence of the reticulum and rumen.

Stretch of the reticulo-rumenal fold together with stretch of the reticulum proved a potent stimulus to both reticulum and rumen contractions. This stimulus was delivered with an ovoid balloon tied into the free pole of the reticulum; on distension this balloon pushed on the reticulo–rumenal fold or extended through the reticulo-rumenal orifice and stretched the fold. By being able to change the shape of the balloon it was possible to compare the effects of its distension when it was spherical and stretched the reticulum alone and when, in addition, it stretched the reticulo-rumenal fold. Direct observation of the position of the balloon and of the activity of the reticulum and associated structures through fistulae permitted a relationship to be established between the record obtained from the water manometer to which the balloon was connected and the activity of the reticulum, recticulo-rumenal fold and adjacent wall of the rumen on which the balloon impinged. The water manometer records revealed that the first response to this stimulus was sometimes a monophasic contraction of the reticulum, followed by a slight contraction of the dorsal sac of the rumen. Subsequent records obtained with the water manometer were of diphasic contractions of the reticulum on which were superimposed, and to which were added, those of the reticulo-rumenal fold and adjacent regions of the wall of the rumen, mainly of its anterior wall near the cardia (Fig. 3).

The criteria used to judge the efficacy of this stimulus and to compare it with stretch of the reticulum alone were the rapidity with which the rumenal contractions developed to a maximum, the duration of the responses and the frequency of the contractions. In an individual experiment it was found that, when the same volume was used to distend a balloon which stretched both the reticulum and the reticulo-rumenal fold or orifice, the rumenal contractions evoked developed more rapidly, were often, as also were reticulum contractions, more frequent, reached a greater height and endured longer, than did the rumen contractions less regularly produced by stretch of the reticulum alone (Figs. 2*a*, *b*, 3). The difference was further accentuated in those experiments in which there were reticulum contractions without any of the rumen on simply stretching the reticulum and the appearance of contractions of the rumen following those of the reticulum only when both the reticulo-rumenal fold and the reticulum were stretched.

Identification of the stimulus as stretch of the reticulo-rumenal fold together with stretch of the reticulum on distension of the elongated balloon projecting towards the rumen from the reticulum depended on different points. The first was that in five decerebrate preparations in which large rumen fistulae were made the reticulo-rumenal fold was stretched manually. This was succeeded by contractions of the reticulum, reticulo-rumenal fold and anterior part of the dorsal sac of the rumen.

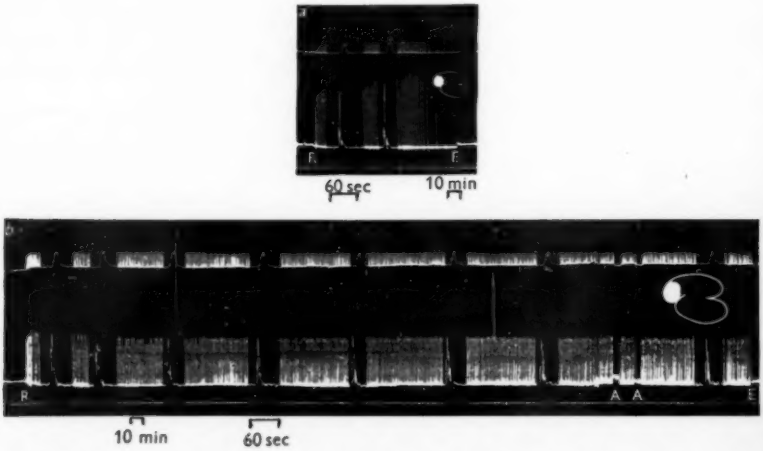

Fig. 2. Decerebrate sheep 13 kg. Comparison of the effects on the reticulum and rumen of (*a*) distension with 225 ml. of a spherical balloon in the reticulum and, (*b*) distension with 225 ml. of an ovoid balloon which extended from the reticulum to distend the reticulo-rumenal orifice. The distension of the balloon in each case is marked R, the point of emptying E. In (*a*) the response continued for 61 min; in (*b*) the water was removed from the balloon after 452 min. A,A show the effects of the introduction of 300 and of 150 ml. of saline into the abomasum. Sequence of records from above downwards: posterior dorsal rumen (frontal-writing lever), reticulum (water manometer), signal, time marker, 60 sec during slow drum speeds, 10 sec during fast drum speeds. The inset shows the position of the balloon in each case.

In these experiments the stretch was thought to be localized to the reticulo-rumenal fold and not to involve the wall of the rumen near the cardia, the other region affected by a balloon projecting through the reticulo-rumenal orifice. The second was obtained from observations with the introduction of the balloon through a cannula inserted into the free ventral pole of the reticulum so as to permit its movement from the reticulum, into the reticulo-rumenal orifice and also through the reticulo-rumenal orifice into the anterior dorsal rumen immediately adjacent to the reticulo-rumenal fold. Records obtained after the distension of the

balloon in these three situations revealed that stretching of the reticulum alone was a weak stimulus to rumen contractions, stretching of the reticulo-rumenal fold as well as of the reticulum proved a strong stimulus whereas distension of the balloon in the anterior part of the dorsal sac of the rumen under these conditions failed to evoke either reticulum or rumen contractions (Fig. 3), when it no longer affected the reticulo-rumenal orifice.

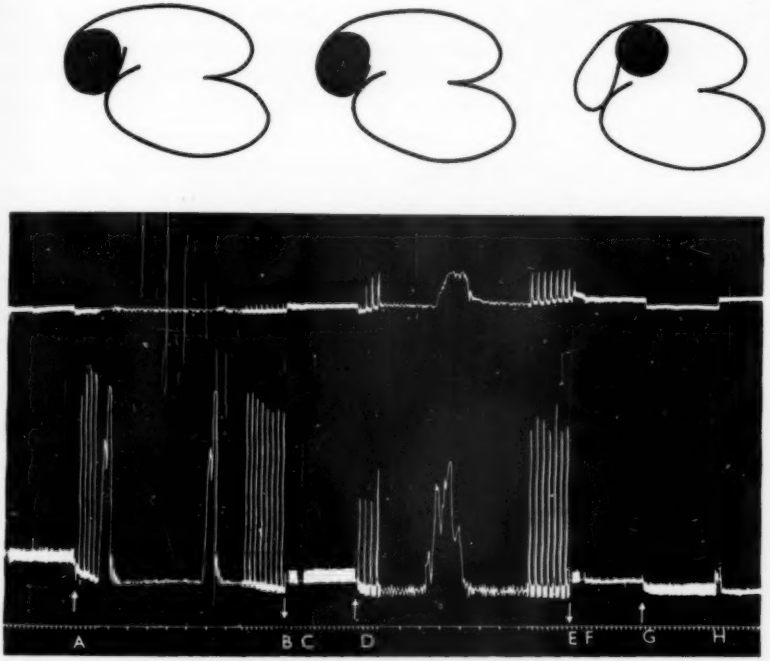

Fig. 3. Decerebrate sheep 15 kg. Sequence of records as in Fig. 2. Time marker 60 sec during slow, 10 sec during fast drum speeds. The record shows the effects on the reticulum and rumen of the distension of a balloon with 250 ml. of water when it was wholly in the reticulum (A–B), when it projected from the reticulum through the reticulo-rumenal orifice (D–E) and in the anterior rumen (G–H). A marks the introduction of 250 ml. of water into the reticulum balloon introduced into it through a reticulum cannula; the water was removed at B. C indicates the movement of the balloon so that on distension with 250 ml. of water at D, it extended from the reticulum through the reticulo-rumenal orifice stretching both the reticulum and reticulo-rumenal fold. The water was removed at E. F marks the movement of the balloon through the reticulo-rumenal orifice into the anterior dorsal sac of the rumen, G its distension in this position with 250 ml. water and H its removal. The diagrams above the tracings indicate the three successive positions of the balloon.

The form of the rumen contractions evoked in decerebrate preparations

The contractions of the rumen involved successively the more anterior regions first, with a later spread to more posterior regions of the rumen (Fig. 4). On manual palpation through a large rumen fistula prepared in the posterior dorsal sac of the rumen it was observed that a contraction of the reticulo-rumenal fold occurred during the second phase of the diphasic reticulum contraction. This contraction reached its height after relaxation of the reticulum was complete; it was succeeded by another contraction separated from the first by a partial relaxation of the reticulo-rumenal fold. Accompanying this second phase of the reticulo-rumenal

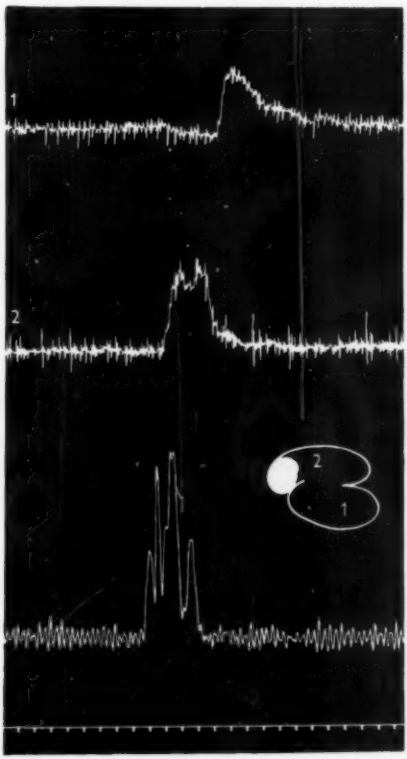

Fig. 4. Decerebrate sheep 24 kg. Showing the sequence of contractions of the posterior ventral blind sac of the rumen, uppermost record (point 1 on inset); mid-dorsal rumen, middle record (point 2 on inset), and the water manometer tracing obtained from a balloon projecting from the reticulum through the reticulo-rumenal orifice as shown in the inset, when distended with 500 ml. water. Time, 5 sec.

contraction was a contraction of the anterior dorsal sac of the rumen which spread progressively posteriorly. The diphasic contractions of the reticulo-rumenal fold were frequently, but not invariably, under these conditions, followed by similarly diphasic contractions of the anterior pillar. These in turn were frequently, but again not invariably, followed by similarly diphasic contractions of the posterior pillar. The contractions of the anterior and posterior pillars interfered with records of the activity of the anterior and posterior ventral sacs of the rumen obtained with the thread-frontal-writing lever recording method when the point of attachment of the thread was close to the pillars. Operative interferences affected the results. Contractions of the posterior dorsal sac were not observed after an incision was made in it. As the effect of the local anaesthetic agent used to make large rumen fistulae acutely wore off, the activity of the rumen became less. This was evident first as a lessening in the regularity and force of contractions of the posterior pillar of the rumen and then of the anterior pillar, the anterior dorsal sac of the rumen and finally of the reticulo-rumenal fold. The contractions of this last-named structure frequently became simple weak monophasic contractions before they ceased.

The effect of other stimuli

Acid in the abomasum. HCl introduced into the abomasum in sufficient strength and volume to lower the pH of the abomasal contents to or below 0·9–1·0 produced reticulum contractions as previously described (Titchen, 1958*a*). Frequently this stimulus could be shown apparently to sum with another stimulus. In Fig. 5*a* the last of a series of contractions of the reticulum and rumen evoked by distension of a balloon in the reticulum had occurred 15 min before, and the response then to acid introduced into the abomasum was a more prolonged and more forceful series of reticulum and rumen contractions than that following acid alone after the reticulum balloon had been emptied (Fig. 5*b*).

Omasal canal stretch was followed by an increase in the frequency of reticulum and rumen contractions evoked by stretch of the reticulum or the reticulum and reticulo-rumenal fold. In one experiment a marked change in the form of contractions of the dorsal sac of the rumen was observed. These become more prolonged and changed from their previously simple form to contractions showing a number of peaks.

Oesophageal stimulation. The passage of a tube (12 mm external diameter) into the oesophagus through the mouth was frequently followed by one or more reticulum or rumenal contractions. In preparations in which reticulum or reticulum and reticulo-rumenal fold stretch had failed to elicit contractions of either the reticulum or rumen this stimulus initiated a series of contractions of the reticulum and rumen which continued after

the stimulus was withdrawn. Distension of a balloon in the oesophagus similarly proved effective under such circumstances, as described by Sellers & Titchen (1959). An increase in the frequency, force and complexity of the rumen and reticulum contractions was observed when the stimulus was applied during the height of a previously established response of these structures.

Abolition of reticulum and rumen contractions. The reticulum and rumen contractions produced as described ceased after the intravenous injection of atropine (0·2–0·5 mg/kg), or section of both vagus nerves in the neck. With greater degrees of stretch of the reticulum, contractions of this structure but not of the rumen, were observed after the dorsal abdominal vagus nerve trunk had been cut in the last part of its course in the thorax

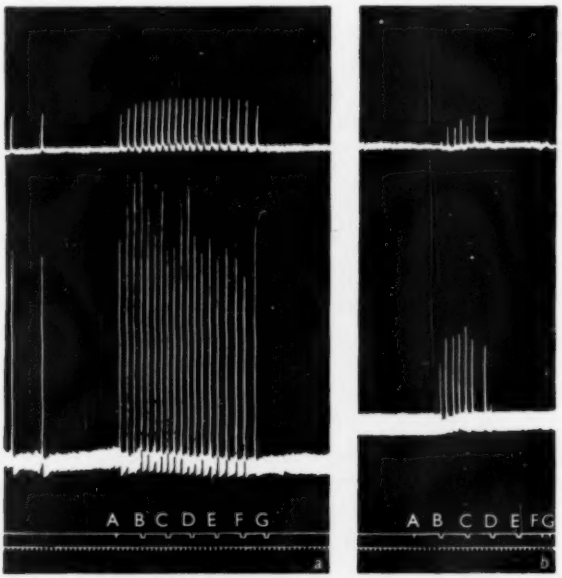

Fig. 5. Decerebrate sheep 13 kg. Records as in Fig. 1. Showing the effects of the introduction of 50 ml. 0·2 N-HCl into the abomasum during the distension with 200 ml. of water of a balloon in the reticulum (*a*) and with the balloon retained in the reticulum but empty of all but about 15 ml. water (*b*). The abomasum had been isolated by ligatures tied at the pylorus and the omaso-abomasal junction. In each case A marks the introduction of the HCl. In *a* the acidity, by titration, of the samples of abomasal contents was B, 0·186N; C, 0·162N; D, 0·134N; E, 0·128N; F, 0·110N. Between F and G the abomasum was washed out with saline and the acidity of the contents removed at G was equivalent to 0·002N. In *b* similarly the acidity of the samples determined by titration was B, 0·175N; C, 0·173N; D, 0·158N; E, 0·142N; F, 0·112N and G, 0·112N. The first of the series of contractions shown in *b* occurred during manipulation of the abomasal cannula when sample B was being taken.

or the first part in the abdomen. Such contractions of the reticulum were weak and ceased after the ventral abdominal vagus nerve had been cut. As previously described by Duncan (1953) electrical stimulation of the peripheral end of the dorsal abdominal vagus nerve evoked stronger contractions of the reticulum and rumen than did that of the ventral abdominal vagus nerve trunk. Contractions elicited thus were annulled by atropine.

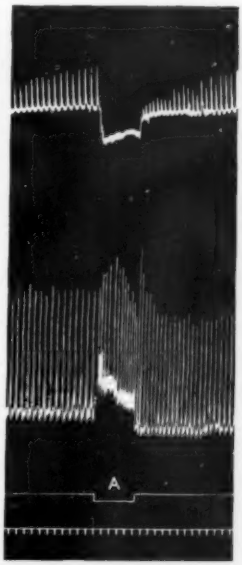

Fig. 6. Decerebrate goat, 11·8 kg. Records as in Fig. 1. Inhibition of rumen contractions on the introduction of 250 ml. saline into the abomasum (A). Reticulum contractions increased in frequency. Stimulus to reticulum and rumen contractions 200 ml. in balloon extending from reticulum through reticulo-rumen orifice. Time 60 sec.

Inhibition

A major experimental difficulty was the ease with which rumen contractions were inhibited. Attempts to extend the observations which involved more extensive dissection led to a failure to elicit contractions of the rumen and a relatively evanescent series of contractions of the reticulum. Once established, contractions of the reticulum and particularly those of the rumen were reduced if a further incision were made in the abdominal wall. These effects were abolished by transection of the spinal cord in its upper thoracic regions, but such preparations rarely survived without the intravenous administration of plasma substitutes and even after these rarely for more than 45–60 min.

More specific inhibitions were those associated with distension of the abomasum or the rumen. Abomasal distension produced a more profound inhibition of rumen than of reticulum contractions, and in one a largely excitatory effect of abomasal distension on reticulum contractions was accompanied by a total inhibition of rumen contractions (Fig. 6). Under these circumstances a prolonged effect on the rumen contractions was seen as a reduction in their force for periods of up to 10 min after removal

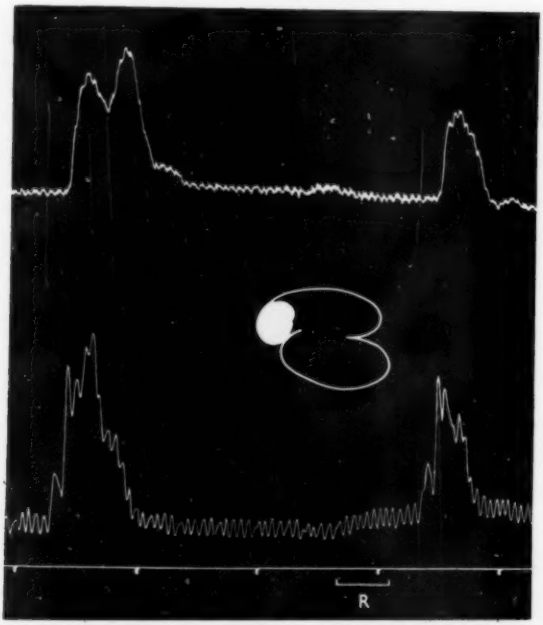

Fig. 7. Decerebrate sheep 15 kg. Dorsal rumen, reticulum contractions, 30 sec time marker (from above downwards). Stimulus to reflex contractions of reticulum and rumen distension with 300 ml. water of a balloon projecting from the reticulum through the reticulo-rumenal orifice. R, insufflation of air into rumen (to a pressure of 10 cm water in the rumen) led to a reduction in the duration and force of the rumen contractions recorded directly from its dorsal sac (upper record) and to a reduction in the deflexions on the water-manometer record associated with rumen contractions.

of the fluid from the abomasum. Both abomasal and rumenal distension produced a reduction in the duration and intensity of rumen contractions (Fig. 7).

Stimulation of the central end of a splanchnic nerve produced an inhibition of rumen and reticulum contractions (Fig. 8). With splanchnic nerve stimulation the degree of inhibition was graded according to the

strength of the stimulus. The inhibition varied from an absolute one to partial inhibition in which there was a marked reduction in the force and duration of rumen contractions, similar to that observed with the partial inhibition resulting from abomasal and rumenal distension.

The inhibitory effects of stimulation of the central end of a splanchnic nerve frequently persisted for some minutes after the stimulus ceased, as described for the reticulum alone (Titchen, 1958*a*).

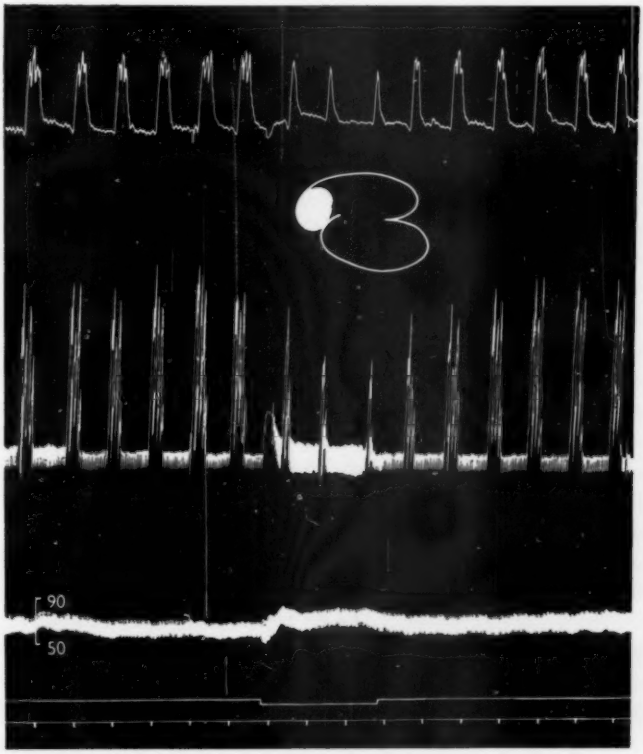

Fig. 8. Decerebrate sheep, effect of stimulation of central end of left splanchnic nerve (signal) for 3 min on contractions of posterior dorsal rumen, reticulum and rumen contractions recorded from balloon distended with 500 ml. water and placed as shown in inset diagram (middle tracing) and on blood pressure. Time 60 sec. Stimulus discharges of a neon tube stimulator, 40/sec, 10 V.

DISCUSSION

One of the most striking features of this series of experiments was the susceptibility of contractions of the rumen to inhibition when compared with those of the reticulum. In both cases much of this inhibition was not

specific but was associated with the preparations for recording movements of, or inducing changes in, the stomach; in general the more extensive the dissection the less was the reflex response obtained. With the reticulum this type of inhibition was evident as a shortening in the duration of the response, whereas with the rumen there was usually an absolute inhibition of its contractions with such a reduced response of the reticulum present. Thus with the rumen the interference of such an inhibition presented a major experimental difficulty in the examination of reflex movements. The efficacy of stretch of the reticulum and reticulo-rumenal fold appeared to become of more significance upon this background of inhibition, particularly when, in addition, its effects were compared with those of stretch of the reticulum alone.

The identification of the stimulus, accorded by a balloon which projected from the reticulum through the reticulo-rumenal orifice, as stretch of the reticulo-rumenal fold was inferred from the experiments in which the effects of distension of a balloon in different loci were compared. This conclusion was confirmed in larger preparations by direct manual stretch of this fold under conditions in which simultaneous stretch of the reticulum was, at least largely, avoided. Furthermore, the possibility that the stimulus delivered by the balloons was of structures other than the reticulo-rumenal fold appears remote in the light of the post-mortem observations that the change consistently observed was of this fold and not of other contiguous structures.

A clear demonstration of the reflex nature of the responses, by separation of the afferent and efferent limbs of the reflex arc such as was provided in the case of the oesophageal groove (Comline & Titchen, 1951) and the reticulum (Iggo, 1956; Titchen, 1958*a*), has not been obtained in the present experiments. Strong inferential evidence for their reflex nature has been obtained from the character of the responses and some of their properties, namely latency, inhibition and summation of stimuli. Taken together with the pharmacological blockade by atropine, the absence of the responses after the vagus nerves had been cut and the presence of the contractions in decerebrate preparations only after the delivery of the appropriate stimuli, these properties provide a strong indication of the reflex nature of the responses. For similar reasons to those advanced previously (Titchen, 1958*a*) a medullary centre is thought to be involved in the case of reflex rumen contractions as well as reticulum contractions.

The close relationship between the control of the contractions of the reticulum and rumen, which might be inferred from the nature of the responses to stretch of the reticulum combined with that of the reticulo-rumenal fold, is not supported by other observations, particularly those on the differential effects of inhibition. In the present experiments a most

consistent observation was that inhibition was more marked on the rumen than on the reticulum. Phillipson (1939) found in conscious sheep that distension of the abomasum led to a more marked inhibition of reticulum than of rumen contractions. Contrary to these observations in the intact animal, the inhibitory effects of distension of the abomasum were more marked on the rumen than on the reticulum in these experiments on decerebrate preparations. This difference was exaggerated by the demonstration in one experiment of complete inhibition of rumen contractions accompanied by increased frequency of reticulum contractions (Fig. 6) during distension of the abomasum. In this one experiment the abomasum had been distended with fluid but there was no evidence of its escape into the omasum or through the pylorus. Such an escape of fluid was associated with variable effects of abomasal distension on reflex reticulum contractions (Titchen, 1958*a*). Movement of fluid from the abomasum into the omasum has been suggested from radiologic observations in intact sheep (Benzie & Phillipson, 1957). The contrary effects of abomasal distension in intact animals and decerebrate preparations may be a reflexion of the greater control over the experimental conditions which is possible in decerebrate preparations.

Evidence obtained from acute experiments indicates that smaller degrees of distension of the abomasum exert their inhibitory effects on reflex reticulum contractions through splanchnic afferents and that after the splanchnic nerves have been cut the same degree of abomasal distension may produce excitatory effects through vagal afferents (Dussardier, 1955; Titchen, 1958*a*). Greater degrees of abomasal distension after section of the splanchnic nerves are inhibitory through vagal afferents (Dussardier, 1955). In the present experiments these observations have not been extended to include the rumen. Stimulation of the central ends of the splanchnic nerves, distension of the abomasum or rumen, all resulted in a marked reduction in the force of rumen contractions and frequently a simplification in their form. This was evident both on the water-manometer records obtained from a balloon projecting from the reticulum through the reticulo-rumenal orifice and on the frontal-writing lever records obtained from threads tied to the wall of the rumen.

The form of the contractions of the rumen associated with those of the reticulum in these experiments appeared to involve successive regions of the rumen in a similar manner to those observed following reticulum contractions in conscious animals in which partial exteriorizations of the reticulum and rumen have been undertaken (Reid & Titchen, 1959 and unpublished observations). As inhibition was manifest as a delay in the onset of rumen contractions and a simplification in their form, and as inhibition was a constant difficulty when a number of laparatomy

incisions were made in order to follow the sequence of rumen contractions, the present observations were limited to those of a more general nature and were not aimed at determining the detailed character or sequence of the contractions of the rumen. They appeared to conform in their general character with those observed in conscious sheep. The absence of contractions of the rumen occurring independently of those of the reticulum in these experiments can be ascribed either to the susceptibility of contractions of the rumen to inhibition, or to the absence in these experiments of appropriate stimuli.

The present experiments provide additional evidence that stimuli arising within the stomach contribute to the regulation of the activity of that organ in ruminants. This evidence, and that produced previously in decerebrate preparations (Iggo, 1956; Titchen, 1958*a*) when taken together with that on the modification of such activity when stimuli are delivered to the stomach of conscious ruminants (Phillipson, 1939; Stevens & Sellers, 1959; Ash, 1959), strengthens the previously advanced contention that the motor activity of the stomach in conscious ruminants may be largely reflex in origin.

SUMMARY

1. Contractions of the rumen, in addition to those of the reticulum, have been demonstrated in decerebrate preparations of sheep and goats under conditions which suggest the reflex nature of these contractions.

2. The contractions were abolished by the administration of atropine and were absent in preparations in which the dorsal abdominal trunk of the vagus nerve had been cut.

3. The most efficacious stimulus to contractions of both the rumen and reticulum was combined stretch of the reticulum and of the reticulo-rumenal fold.

4. Both reticulum and rumen contractions were less regularly elicited by stretch of the reticulum alone, the introduction of acid into the abomasum, oesophageal stimulation and omasal canal stimulation.

5. A major experimental difficulty was inhibition occurring after interferences undertaken in order to obtain records of, or access to, different structures within the abdomen.

6. Inhibition was observed with abomasal distension, rumen distension and stimulation of the central end of the splanchnic nerves.

7. Inhibitory effects of these stimuli were as a rule more marked on the rumen than on the reticulum. In one instance complete inhibition of the rumen contractions was accompanied by increased frequency of reticulum contractions during abomasal distension.

My thanks are due to Dr R. S. Comline for much helpful discussion both during the course of this work and its preparation for publication, to Messrs G. E. Baker and W. A. Whybrow for their technical assistance, to Mr J. Smith for his care of the animals, and Miss A. Howlett for the assistance in the preparation of tracings and diagrams. The preliminary phases of this work were undertaken during the receipt of a grant from the Agricultural Research Council.

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POTASSIUM CHLORIDE MOVEMENT AND THE MEMBRANE POTENTIAL OF FROG MUSCLE

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Hodgkin & Horowicz (1959) have studied the permeability of frog muscle fibres to potassium and chloride. They concluded that the variation of the chloride conductance (g_{Cl}) with membrane potential and external chloride concentration was consistent with the predictions of the constant-field theory (Hodgkin & Katz, 1949), assuming a constant chloride permeability (P_{Cl}). The variation of the potassium conductance (g_K) could not be fitted by the constant-field equations unless the potassium permeability (P_K) was made to vary with the magnitude and direction of the driving force on the potassium ion. Their conclusions were based on potential measurements from isolated muscle fibres which had been equilibrated in various solutions and were subjected to rapid changes of solution. The present experiments, which repeat some of their potential measurements on whole sartorius muscles, were primarily designed to determine the internal concentrations of potassium and chloride at equilibrium in various solutions and to follow the net movements of these ions when the muscle was transferred from one solution to another. The behaviour of muscles in solutions with extra potassium or chloride was first described by Boyle & Conway (1941), and as far as the inward movements and equilibrium concentrations of potassium and chloride are concerned their results are confirmed by the present experiments. However, Boyle & Conway did not examine the outward movement of potassium and chloride from muscles which had been loaded with these ions. In the present experiments most attention has been given to situations where there is a net outward movement of potassium and chloride, since it appears that under these conditions the permeability to potassium is reduced (Hodgkin & Horowicz, 1957; Adrian, 1958). The conditions were chosen so that the driving force on the potassium ion in an outward direction ($V-V_K$) could be altered by changing V or V_K more or less independently. V is the internal potential and V_K is the potassium equilibrium potential and is equal to RT/F ln $f_o[K_o]/f_i[K_i]$. Where the equilibrium potential of an ion has been calculated, it has been assumed that the activity coefficients (f_o and f_i) are equal.

METHODS

All experiments were done on the sartorius muscle of English frogs (*Rana temporaria*). The muscle was usually left attached to the pelvic bone, which was split when paired muscles were required. Up to four pairs of muscles were suspended in a beaker of solution by threads tied to their tibial tendons. The muscles hung from a wire frame moved up and down by a small electric motor to ensure adequate stirring. In experiments involving the washing out of radioactive tracer, the muscle was cut from its pelvic attachment and fine threads tied to a slip of the pelvic tendon as well as to the tibial tendon. The muscle was then tied to a frame made of thin glass rod. All experiments were carried out at room temperature.

The apparatus and methods for the measurement of membrane potential were similar to those described in detail in a previous paper (Adrian, 1956). Micro-electrodes (Ling & Gerard, 1949) filled with 3 M-KCl, with resistances between 5 and 20 MΩ and tip potentials less than 5 mV, were used throughout. The membrane potential was measured by backing off the deflexion of the oscilloscope trace with a calibrator connected between the muscle bath and earth, or by photographic records taken on either stationary or moving film. When the membrane potential was altered by passing current through the membrane, a second micro-electrode connected to a square wave generator was introduced into the fibre close to the potential recording electrode. In order to overcome the non-linear resistance characteristics of the micro-electrode, a resistance of 100 MΩ was placed in series with it.

Solutions

Ringer's fluid. The composition is given at A in Table 1, and is the same as that used previously (Adrian, 1956).

Solutions with extra KCl or NaCl. The composition of solutions with potassium concentrations of 50 and 100 mM, and containing chloride, are given at B and D in Table 1. C and E give the composition of solutions isotonic respectively with B and D, but with the normal potassium concentration.

TABLE 1. Composition of solutions

Solution	K^+	Cl^-	Na^+	Ca^{2+}	HPO_4^{2-}	$H_2PO_4^-$	SO_4^{2-}	Sucrose (m-mole/l. solution)	Relative tonicity
			(mg ion/l. solution)						
A	2·5	121·1	120	1·8	2·15	0·85	—	—	1·0
B	50	168·6	120	1·8	2·15	0·85	—	—	1·37
C	2·5	168·6	167·5	1·8	2·15	0·85	—	—	1·37
D	100	218·6	120	1·8	2·15	0·85	—	—	1·77
E	2·5	218·6	217·5	1·8	2·15	0·85	—	—	1·77
F	100	0	98	6·3	1·08	0·43	104	165	1·77

Rounded values of 120, 170 and 220 mM have been used when the chloride concentration of a solution is referred to in the text.

Sulphate solution containing 100 mM-K. The composition of this solution, which is chloride-free and isotonic with solutions D and E, is given at F (Table 1). $CaSO_4$ was added, as described by Hodgkin & Horowicz (1959), to increase the concentration of ionized calcium towards its normal level. In a previous paper (Adrian, 1956) sulphate solutions were made up with 1·8 mM-$CaCl_2$, and it was noted that muscles in these solutions twitched spontaneously when the potassium concentration was less than 10 mM.

Estimation of intracellular ions

Sodium and potassium. These ions were estimated with a flame photometer. The method has been described previously (Adrian, 1956).

Chloride. Two methods were used, one using the isotope ^{36}Cl and the other an electrometric titration based on the method of Sanderson (1952).

Method (1). Provided the extracellular space is known, the total radioactivity of a muscle soaked in a solution containing ^{36}Cl of known specific activity can be used to estimate the internal concentration. Muscles were therefore soaked for 10 min in a normal Ringer's fluid containing ^{36}Cl, during which time the extracellular chloride should be very nearly completely exchanged. They were then transferred to 1 ml. of inactive Ringer's solution. The extracellular space of the muscle was determined from the specific activity of the soaking solution and the amount of radioactivity washed out of the muscle. As these muscles were to be used later to estimate the internal chloride concentration in a solution with raised KCl concentration, they were not blotted after the initial 10 min period in radioactive Ringer's solution, in order to avoid any damage to superficial fibres. Instead, they were drained by allowing them to touch the side of the tube in which they had been soaked. The fluid adhering to the surface of the muscle and the uncertainties of the draining procedure probably account for the size and variability of the extracellular space determined in this way (see below). Some exchange of intracellular chloride will have taken place in the 10 min period, but since the ratio of the quantities of extra- to intracellular chloride in Ringer's solution is about 16:1, only a small error should be introduced into the estimate of the extracellular space by this exchange.

After the extracellular ^{36}Cl had been washed out, the muscle was transferred to a second radioactive solution with a raised concentration of KCl and a known specific activity of chloride. The muscle was removed from this solution after a variable length of time and either immediately, or after a further period in a solution with the normal potassium concentration but the same chloride specific activity, was drained and weighed without blotting. After drying the muscle was weighed, and then extracted in the cold with distilled water and a final 1 ml. of inactive Ringer's solution. The counting rate of the remains of the muscle after this treatment was not distinguishable from background. The chloride in the extract was precipitated as AgCl and counted with a mica end-window G–M tube. A detailed description will be published elsewhere. Initially trouble was caused by protein in the extracts of dried muscle, which interfered with the coagulation of the AgCl precipitate. Unless the protein was precipitated with trichloroacetic acid, the AgCl remained so finely divided that some of it passed through the filter-paper disks on which it was spread for counting. Self-absorption in the precipitate could not be eliminated, so that it was important that the conditions for precipitation and counting should be kept as uniform as possible. The total quantity of precipitate was kept virtually constant, and when the specific activity of the soaking fluid was determined a dried but inactive muscle was added to the tube containing a weighed quantity of radioactive solution (15–30 mg). These tubes were put through the same extraction procedure as those containing muscles loaded with ^{36}Cl.

Method (2). Electrometric titration of chloride was used on all muscles which had been treated with solutions containing 100 mM-K. The dried muscles were put into 5 ml. of distilled water, which was evaporated to 1·5 ml. in an oven at 95° C. After cooling, 2·5 ml. of 50 % glacial acetic acid was added to the muscle extract. The chloride in the extract was titrated with 0·01 N-$AgNO_3$. The end-point was determined by measuring the potential difference between an Ag-AgCl spiral in the extract and a bright platinum wire extending through and sealed into the wall of the burette just below the tap. The $AgNO_3$ solution was added in steps of 0·1 or 0·05 ml. and the potential measured after each addition. If the potential is plotted against the volume of solution added, a sigmoid curve is obtained, and the end-point was taken as the middle of the steep central portion of the curve. The $AgNO_3$ solution was standardized against a NaCl solution of known concentration. Successive titrations of 1 ml. of the standard NaCl solution (containing about 6 μmole NaCl) were reproducible to within 2 %.

The adequacy of the extraction with hot distilled water was tested in two ways. A second

extraction of the muscle produced no measurable quantity of chloride in the extract. To exclude the possibility that some chloride remained tightly bound to the muscle, two muscles were loaded with ^{36}Cl by soaking in a radioactive solution containing 100 m-mole/l. of added KCl. The distilled water extract and the muscle remains were counted separately. The radioactivity left in the muscle was about 0·5 % of the radioactivity in the extract, and it corresponded to the small quantity of extract which adhered to the muscle on its removal from the extract.

In general, either the chloride or the potassium and sodium content was determined for a single muscle. Paired muscles from the same frog were sometimes treated identically and one used for chloride and the other for sodium and potassium estimations. However, in some experiments the chloride, potassium, and sodium contents were determined for both members of a pair of muscles which had been treated differently. The dried muscles were put into weighed platinum crucibles and extracted in hot distilled water. The final weight of extract was obtained (approx. 2 ml.). As much as possible of the extract was removed from the crucible and divided into two portions; 1·8 ml. (approx.) was used for chloride estimation, and 0·2 ml. (approx.) for estimation of K and Na in the flame photometer after dilution with about 20 ml. of distilled water. To check whether any Na or K remained bound to the muscle, the remnants of the muscle were ashed and estimated in the usual way. There was no evidence that more Na or K remained in the muscle after extraction than could be accounted for by the small quantity of extract left in the crucible after removing the portions for chloride and cation estimations.

RESULTS

Extracellular space

The largest uncertainty in any estimates of the intracellular concentration of muscle is introduced by the extracellular space, for which allowance must be made. The most widely accepted figure for the extracellular space of blotted sartorius muscle is 12·5 % (Desmedt, 1953). It is interesting to compare the extracellular space for unblotted but drained muscles, obtained from a 10 min soaking in Ringer's solution containing ^{36}Cl. The average extracellular space, by this method, was $29{\cdot}1 \pm 0{\cdot}78$ % (18 muscles; ±S.E.M.), and the average dry-to-wet-weight ratio of these muscles was $16{\cdot}6 \pm 0{\cdot}25$ %. If it is assumed that these muscles would have had an extracellular space of 12·5 %, had they been blotted, then they would have had a dry-to-wet-weight ratio of $20{\cdot}5 \pm 0{\cdot}2$ %. This differs significantly from the mean dry-to-wet-weight ratio of blotted muscles which have been in a normal Ringer's solution ($21{\cdot}5 \pm 0{\cdot}15$ %). A systemic error of −10 % in the estimate of the extracellular space in the unblotted muscles would account for the difference, but the possibility that blotting a muscle removes some of the intracellular water as well as the surface water cannot be excluded.

Despite these uncertainties, the extracellular space was taken to be 12·5 % for blotted muscles whose dry-to-wet-weight ratios were in the normal range. Many of the experiments involved muscles which had lost water from the fibres, so that it was important to know how the extracellular space varied with the volume of the fibres. The extracellular spaces

of eight muscles were determined. Four were soaked in a sulphate solution containing 100 mM-K (solution F, Table 1) and four in a similar solution from which some of the sucrose and Na_2SO_4 had been removed. The sucrose in each muscle was determined colorimetrically (Lewis, 1952). Paired muscles which had been soaked in solutions containing no sucrose and isotonic with Ringer's solution were used for blank determinations. By estimating the sucrose in several dilutions of the soaking solution a graph was obtained from which the number of milligrams of such a solution in each muscle could be read directly. The results are shown in Table 2.

TABLE 2. Variation of the extracellular space

$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	Extracellular space (%)	Space calculated on constant volume assumption (%)
24·7	17·4	14·4
25·3	13·5	14·7
25·4	15·9	14·8
26·0	15·2	15·1
Mean 25·3	15·5 ± 0·8	14·8
29·6	20·0	17·2
30·7	18·8	17·9
31·2	16·7	18·1
31·6	20·3	18·4
Mean 30·8	18·9 ± 0·8	17·9

Although there is considerable scatter it is obvious that the percentage space does not remain constant as the fibres shrink. The results are closer to those that would be predicted if the volume of the extracellular space remained constant while only the fibres shrank. The extracellular space was therefore calculated for shrunken muscles from the dry-to-wet-weight ratio assuming a constant *volume* of extracellular space, such that if the dry-to-wet-weight ratio had been 21·5% the percentage of extracellular space would have been 12·5.

The gain of potassium and chloride in solutions of high KCl concentration

The net movement of chloride into muscles in solutions containing 50 and 100 m-mole KCl/l. in addition to the other constituents of Ringer's solution at their normal concentrations (solutions B and D, Table 1) was followed by analysing muscles at varying times after their transfer from Ringer's to the new solution. The concentration of chloride in the fibre water is plotted against time in Fig. 1. The internal concentration of chloride in Ringer's solution is taken to be 3·8 m-mole/kg fibre water (Adrian, unpublished). After an initial rise the internal chloride concentration reaches a new steady level. Table 3 gives the new steady-

state values for the internal potassium and chloride concentrations in the two solutions. The increases in the concentrations of potassium and chloride are more or less equal in the extracellular and intracellular water. At high external potassium chloride concentrations the internal and external

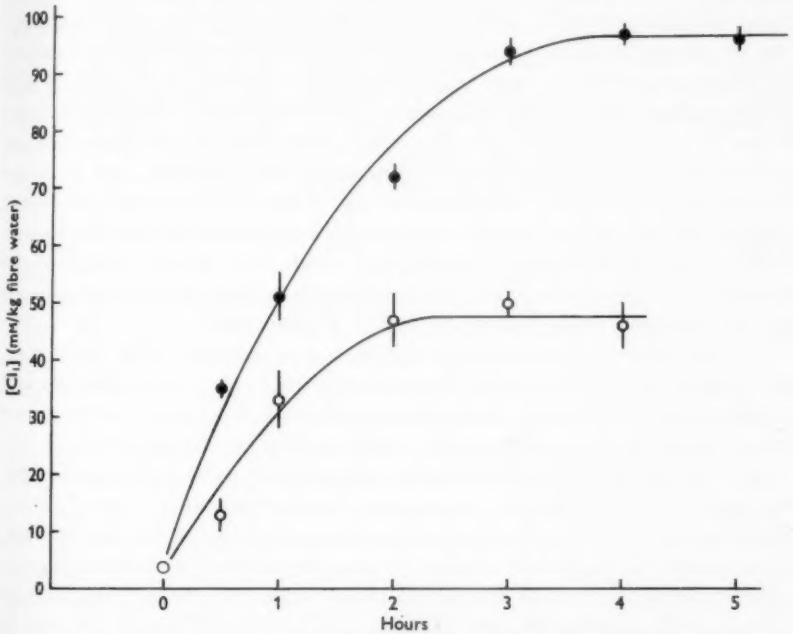

Fig. 1. The change of internal chloride concentration after transferring a muscle from Ringer's to solutions with extra KCl. Open circles, muscles transferred to solution B (50 mM-K, 170 mM-Cl); solid circles, muscles transferred to solution D (100 mM-K, 220 mM-Cl). Vertical lines are $\pm$ one standard error of the mean.

TABLE 3. Steady-state internal concentrations (mean $\pm$ S.E.M.) in solutions of varying KCl concentration

External solution	[K_i]	[Cl_i] (m-mole/kg fibre water)	Δ[K_i]	Δ[Cl_i]	$\frac{\text{dry wt.}}{\text{wet wt.}} \times 100$
Ringer*	139 $\pm$ 2·0	3·8			21·5 $\pm$ 0·15 (14)
Solution B,† 50 mM-K, 170 mM-Cl	192 $\pm$ 2·9 (6)	47 $\pm$ 1·9 (13)	+53	+43	23·0 $\pm$ 0·28 (6)
Solution D,‡ 100 mM-K, 220 mM-Cl	242 $\pm$ 3·5 (14)	97 $\pm$ 1·2 (16)	+103	+92	23·5 $\pm$ 0·21 (22)

Figures in brackets are the number of muscles estimated. * Values for Ringer taken from Adrian (1956, and unpublished). † Results from 2–4 hr in solution B pooled. ‡ Results from 4–5 hr in solution D pooled.

potassium chloride products do not differ by more than 6%, though the changes in concentration of potassium and chloride inside the muscle fibre differ by more than this amount. Such an effect would be expected if the valence of the internal impermeable anion was not unity, and a small decrease in volume in the new steady state would be required to make the quantities of K^+ and Cl^- entering the muscle equal (see Discussion). The dry-to-wet-weight ratios given in the last column of Table 3 indicate that there is a small decrease in volume in the solutions with a raised KCl concentration. The results presented in Fig. 2 and Table 3 agree with those of Boyle & Conway (1941), who first showed that frog muscle fibres behave as if they are freely permeable to potassium and chloride, and that the internal and external KCl products are equal when the external potassium chloride concentration is high. It is worth while stressing the agreement between the present results and those of Boyle & Conway, in view of the fact that their results have not been confirmed in some recent experiments on toad muscle (Shaw, Simon, Bennet & Muller, 1957).

On transferring a muscle from Ringer's to a solution with extra KCl, but normal NaCl, content the muscle shrinks initially. As potassium and chloride enter with water, the muscle swells until it reaches a volume just less than the volume in Ringer's solution. During the period when the volume of the muscle is changing, the rate of change of internal concentration cannot be used directly to assess the net inward movement of an ion. However, the quantity of solids in a muscle can be presumed to be constant, and a measure of the net movement can be obtained by expressing the concentration in terms of dry weight rather than fibre water. The average rate of entry of chloride over the first half hour in the solution with a potassium concentration of 50 mM is 130 m-mole/kg dry wt./hr, and in the solution with a potassium concentration of 100 mM the rate of entry is 142 m-mole/kg dry wt./hr.

The loss of potassium and chloride into chloride solutions from muscles loaded with KCl

After 3 hr in a solution which contains 50 m-mole KCl/l. in addition to the other constituents of Ringer's solution at their normal concentrations (solution B), the muscle fibres contain approximately 50 m-mole/kg fibre water of K^+ and Cl^- in excess of the internal concentrations of these ions in Ringer's solution. If muscles, loaded with KCl in this way, are returned to Ringer's solution, how rapidly is the extra KCl lost? The open circles in Fig. 2 show the internal chloride concentration in m-mole/kg fibre water at varying times after the loaded muscles are transferred back to Ringer's solution. The concentration shows an initial rapid drop and this is followed by a slower return towards a low value. Since the fibres have reached

osmotic equilibrium with the high KCl solution, which is hypertonic to Ringer's, the fibres would be expected to swell when they are transferred back to Ringer's solution, and a drop of internal concentration would result from water movement alone. The mean internal chloride concentration, expressed in terms of the dry weight of the muscle, is $155 \pm 6{\cdot}1$ m-mole/kg dry wt. in the high-KCl solution ($\pm$ S.E.M.; 13 muscles at 2, 3 and 4 hr in solution B). Half an hour after the transfer to Ringer's solution the internal chloride concentration in the same units is 163 ± 17 m-mole/kg dry wt. The initial rapid drop of chloride concentration in m-mole/kg fibre water is therefore due to the inward movement of water, and there appears to be a negligible outward movement of chloride in the first half hour.

If the muscles which have been loaded with KCl in the solution with a potassium concentration of 50 mM are transferred to a fluid which is isotonic with the loading solution, there will be no rapid movement of water. The composition of such a solution is given at C in Table 1. It has the same potassium concentration as Ringer's and the extra KCl has been replaced by NaCl. In this solution potassium and chloride would be expected to leave the fibre until the external and internal KCl products were again equal. There would also be a slow movement of water with the KCl in order to keep the fibre contents isotonic with the outside solution. Half an hour after the transfer of loaded muscles from the high-KCl solution to a solution isotonic with it but with normal potassium concentration, the internal chloride concentration has not fallen, nor has the concentration in m-mole/kg dry weight altered ($153 \pm 8{\cdot}0$ m-mole/kg dry wt.). Both in Ringer's and in the hypertonic solution, although one would expect a substantial loss of both potassium and chloride, no measurable loss of chloride occurs in the first half hour. Subsequently a slow loss does occur at a rate of about 40 m-mole/kg dry wt./hr.

After 4 hr in a solution which contains 100 m-mole KCl/l. in addition to the other constituents of Ringer's solution at their normal concentration (solution D), the muscle fibres contain approximately 100 m-mole/kg fibre water of potassium and chloride in excess of their internal concentrations in Ringer's. Muscles in this condition were transferred to a solution isotonic with the loading solution but with a potassium concentration equal to that of Ringer's (solution E). The chloride concentrations of the two solutions were equal (220 mM). The changes in the internal concentrations of chloride, potassium, and sodium were followed by analysis. Table 4 gives the results from eight pairs of muscles in detail. One of each pair was analysed for all three ions at the end of the initial equilibration in the high-potassium solution (D), and the other was analysed at the end of a further 2 hr in the low-potassium solution (E). Comparison of pairs

of muscles shows that the internal chloride concentration may fall to half its initial value in the 2 hr in the low-potassium solution, but in several muscles it has fallen by much less. There is also a variable fall in the potassium concentration, and a roughly complementary rise in the sodium concentration. The quantities of these three ions leaving and

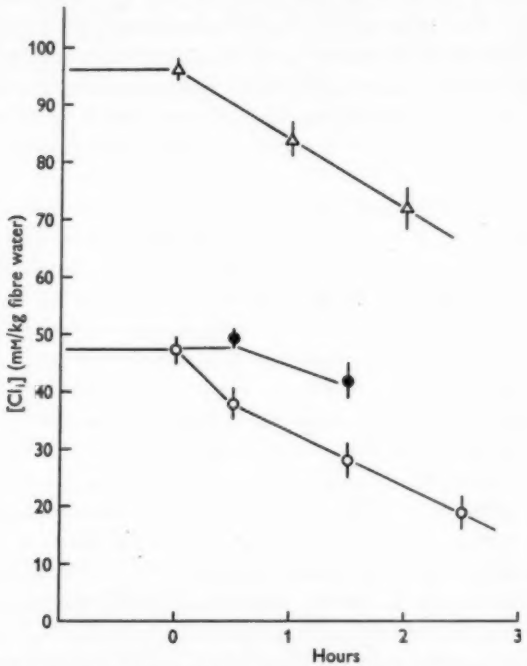

Fig. 2. The change of internal chloride concentration after transferring muscles from solutions with extra KCl to solutions with the same potassium concentration as Ringer's (2·5 mM-K). Open circles, muscles transferred to Ringer's (2·5 mM-K, 120 mM-Cl) after equilibration in solution B (50 mM-K, 170 mM-Cl); solid circles, muscles transferred to solution C (2·5 mM-K, 170 mM-Cl, isotonic with B) after equilibration in solution B; open triangles, muscles transferred to solution E (2·5 mM-K, 220 mM-Cl, isotonic with D) after equilibration in solution D (100 mM-K, 220 mM-Cl). Vertical lines are ± one standard error of the mean.

entering the fibres can be calculated from the concentration changes and the change in the dry-to-wet-weight ratio for each pair of muscles. One would expect that the loss of chloride would equal the total loss of cation (loss of potassium minus the gain of sodium). For the eight pairs of muscles in Table 4 the mean value for the difference between the loss of chloride and the loss of cation was not significantly different from zero (3·2 ± 5·3 m-mole/kg fibre water; mean ± S.E.M.).

The internal concentrations of muscles loaded in the high-KCl solution (D, 100 mM-K) and soaked for 1 hr in the low-potassium solution (E, 2·5 mM-K) were: chloride, $84 \pm 3{\cdot}0$ m-mole/kg fibre water (10 muscles); potassium, $222 \pm 7{\cdot}3$ m-mole/kg fibre water (8 muscles); sodium, $35 \pm 7{\cdot}0$ m-mole/kg fibre water (8 muscles). After 2 hr in the low-potassium solution (E) the internal concentrations were: chloride, $77 \pm 3{\cdot}5$ m-mole/kg fibre water; potassium, $216 \pm 4{\cdot}5$ m-mole/kg fibre water; sodium, $34 \pm 3{\cdot}7$ m-mole/kg fibre water (each value is the mean of 16 estimations, which include the data of Table 4; ± S.E.M.). During the first 2 hr in the low-potassium solution chloride is lost at the rate of 36 m-mole/kg dry wt./hr.

TABLE 4

Time in solution D, 100 mM-K, 220 mM-Cl (hr)	Time in solution E, 2·5 mM-K, 220 mM-Cl (hr)	$\frac{\text{dry wt.}}{\text{wet wt.}} \times 100$	$[K_i]$	$[Na_i]$	$[Cl_i]$
			(m-mole/kg fibre water)		
4	0	23·2	236	7	100
4	2	24·2	221	20	84
4	0	22·6	229	7	96
4	2	23·7	230	15	94
4	0	22·7	226	18	102
4	2	24·0	220	25	65
4	0	22·7	233	21	96
4	2	24·2	222	25	82
4	0	22·4	229	14	103
4	2	24·1	218	30	91
4	0	22·6	249	17	95
4	2	24·7	207	31	71
4	0	23·2	240	9	92
4	2	26·4	197	40	57
4	0	21·7	238	12	101
4	2	25·7	204	49	48

Brackets indicate muscles from the same frog.

Potential changes accompanying loss of KCl into chloride solutions

The low rate of outward movement of chloride from muscles loaded with KCl in solutions with high potassium and chloride concentrations (B and D) and transferred to solutions with a normal potassium concentration (Ringer's, C and D) could be caused by a very low chloride permeability or by a very small driving force on the chloride ion under these conditions. If the permeability to chloride was low, the membrane potential would be sensitive to the ratio of the internal and external potassium concentrations, and the fibre would be expected to repolarize when it was transferred from the high- to the low-potassium solution. If, on the other hand, the potassium permeability was low in comparison with the chloride permeability, the change in potential might be small because the chloride

concentrations of the high- and low-potassium solutions are equal. Hodgkin & Horowicz (1957) reported that the membrane potential of single fibres, equilibrated in a Ringer's fluid with extra KCl, is insensitive to a reduction of the external potassium concentration, but that the membrane behaves as if it were a chloride electrode when the external chloride concentration is reduced.

The membrane potential of the surface fibres of a sartorius muscle was measured after 3 hr in the solution with a potassium concentration of 50 mM (solution B; chloride concentration 170 mM). The mean internal potential was $-30{\cdot}5 \pm 0{\cdot}2$ mV (S.E.M., 48 fibres from three muscles). The

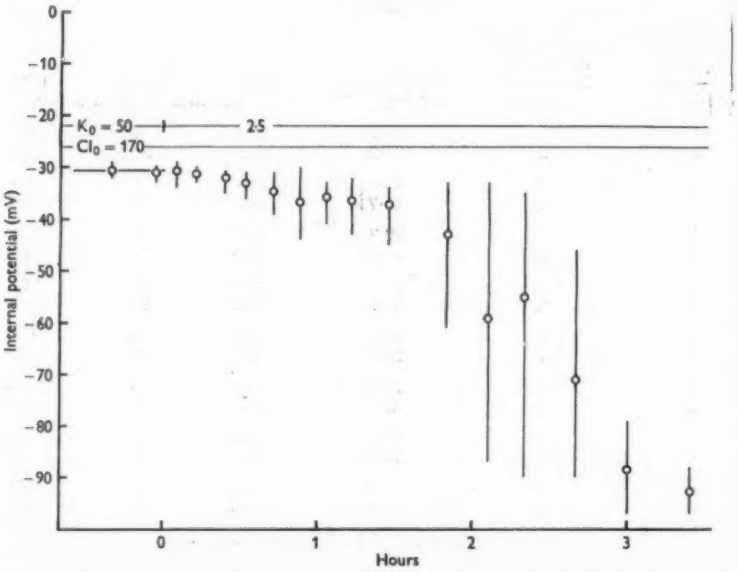

Fig. 3. The repolarization of muscle fibres which have been loaded with KCl in solution B (50 mM-K, 170 mM-Cl) and transferred to solution C (2·5 mM-K, 170 mM-Cl). The circles are the means of groups of 8–10 measurements and the vertical lines join the highest and lowest measurement in each group.

calculated value of the chloride equilibrium potential under these conditions is $-32{\cdot}4$ mV ($V_{Cl} = RT/F$ ln 47/170). The muscle was then transferred to the solution with potassium and chloride concentrations of 2·5 and 170 mM respectively (solution C), and the membrane potential was measured as soon as possible after the transfer and then at approximately 10 min intervals. The results of one experiment are shown in Fig. 3, where the circles represent the means and the lines join the highest and lowest values of each group of measurements. It is obvious that no immediate

repolarization was detectable, and that for the first 1½ hr repolarization was very slow. After that repolarization was rapid, and the time course of repolarization must have been steeply S-shaped in any individual fibre. If during the period when potassium and chloride are leaving the fibre, the potassium permeability is limiting the rate of loss (the absence of any measurable repolarization strongly suggests that this is the case initially), then the membrane potential will be close to the chloride equilibrium potential at any moment, and the S-shaped time course suggests that KCl leaves each fibre slowly at first, then more rapidly, and slowly again when the fibre is nearly fully repolarized.

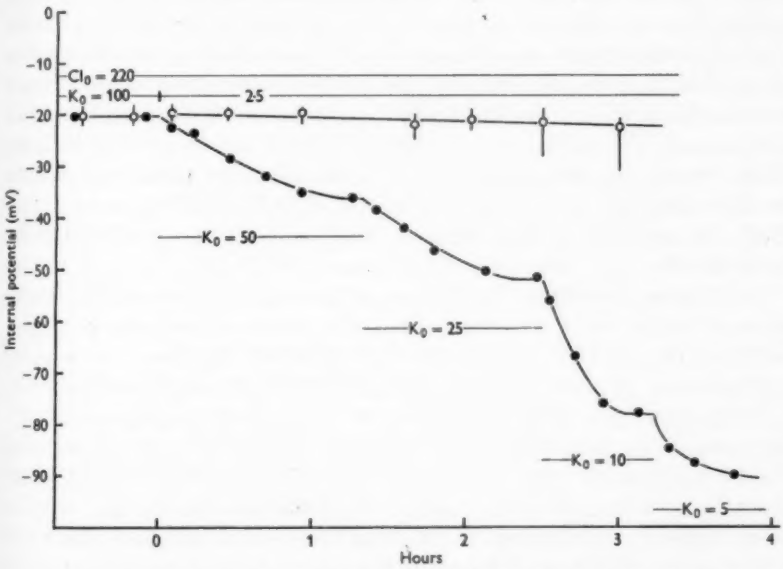

Fig. 4. The repolarization of muscle fibres loaded with KCl in solution D (100 mM-K, 220 mM-Cl) and transferred to solution E (2·5 mM-K, 220 mM-Cl) (open circles), and to mixtures of solutions D and E with progressively falling potassium concentrations (solid circles). The circles are the means of groups of 8–10 measurements and the vertical lines join the highest and lowest measurement in each group.

Figure 4 gives the results of two experiments in which the membrane potential was measured after 4 hr in the solution with 100 mM-K and 220 mM-Cl (solution D). It was again measured after transfer to solutions with the same chloride concentration but different potassium concentrations. The internal potential in the solution with 100 mM-K was $-20{\cdot}2 \pm 0{\cdot}14$ mM (S.E.M., 78 measurements from seven muscles) which was close to the

calculated equilibrium potential for both potassium and chloride ($V_K = -22{\cdot}3$ mV, $V_{Cl} = -20{\cdot}6$ mV). The open circles in Fig. 4 are from a muscle which was transferred to a solution with 2·5 mM-K and 220 mM-Cl (solution E). Provided the surrounding solution is kept well stirred, none of the surface fibres repolarizes by more than a few millivolts in the course of 3 hr. The solid circles are from a muscle which was transferred to a solution with 50 mM-K and 220 mM-Cl, and then successively to solutions with the same chloride concentration but with 25, 10 and 5 mM-K. After each transfer, though the immediate change in potential was small, the membrane potential repolarized towards the potassium equilibrium potential in each solution. This experiment shows that, even after 4 hr in 100 mM-K, muscle fibres are capable of repolarizing provided that the external conditions are appropriate. It also shows that the repolarization and the loss of extra potassium and chloride are faster when the difference between the membrane potential and the potassium equilibrium potential is kept small. For the change of external potassium concentration from 100 to 2·5 mM, the driving force on the potassium ion immediately after the change is 95 mV ($V = -20$ mV; $V_K = RT/F \ln 2{\cdot}5/240 = -115$ mV). When the external potassium concentration is changed from 100 to 50 mM the driving force is only 17 mV.

The internal chloride concentration of the two muscles in Fig. 4 was measured at the end of the experiments. The muscle which had repolarized contained 19 m-mole/kg fibre water and the other contained 65 m-mole/kg fibre water. If all the fibres of this muscle had contained 65 m-mole Cl/kg fibre water, the surface fibres should have repolarized to -31 mV. However, if the deep fibres had lost more chloride than the superficial fibres this difficulty would disappear, since the estimated chloride concentration is averaged over all the fibres of the muscle. At first sight it seems unlikely that the deep fibres should recover more rapidly than the surface fibres. However, diffusion of potassium in the extracellular space will make the external potassium concentration deep in the muscle higher than at the surface of the muscle, so that the driving force on the potassium ion across the membrane of the deep fibres will be less than that for the superficial fibres, and the loss of potassium and chloride from the deep fibres will therefore be more rapid.

The loss of potassium and chloride in sulphate solutions from muscles loaded with KCl

In the cases considered so far, after first loading the muscle with KCl in a high-potassium solution, the external potassium concentration has been reduced and the external chloride concentration held constant. Under these conditions the membrane potential remains very nearly

constant despite the fact that V_K is far removed from it. Hodgkin & Horowicz (1959) have shown that lowering the external chloride, after loading fibres with KCl, can cause the internal potential to become as much as 70 mV positive. If the external potassium concentration is left constant and the chloride reduced, V_K is unchanged but the membrane potential is altered so that the driving force on both potassium and chloride is again in an outward direction.

Muscles containing approximately 100 m-equiv/l. of potassium and chloride in excess of their concentrations when in Ringer's solution, prepared by soaking in a high-KCl solution (solution D; 100 mM-K, 220 mM-Cl) were transferred to a chloride-free sulphate solution with a potassium concentration of 100 mM (solution F, Table 1). The internal concentrations were measured in the usual way, and Table 5 gives the internal concentrations at varying times after the transfer, and for muscles which had been transferred from Ringer's directly to the chloride-free solution. In the course of 3 hr most of the extra chloride and potassium are lost, and the muscles approach the same internal composition as those muscles which had been put directly into the chloride-free sulphate solution. Though the muscles loaded with KCl lose potassium in the chloride-free solution, their internal potassium concentration does not change, because the fibres shrink as potassium and chloride are lost.

Potential changes accompanying loss of KCl into chloride-free sulphate solution

If the membrane potential of the surface fibres of a muscle which has been loaded with KCl in a high-KCl solution (solution D; 100 mM-K, 220 mM-Cl) is measured shortly after transfer to the chloride-free sulphate solution (solution F; 100 mM-K, 0 mM-Cl), it is found that the potential of the inside of the fibre is 60 mV positive to the outside. An experiment of this type is illustrated in Fig. 5. Over the first hour the internal potential slowly declines to about +40 mV, but between 60 and 70 min after the change of solution the internal potential of a few fibres returns to −20 mV. The proportion of fibres with internal potentials of about −20 mV increases at the expense of those with potentials of about +40 mV, until at the end of 2 hr the potential of all the surface fibres is −20 mV. During the second hour no fibres with internal potentials between +30 and −18 mV are seen; each fibre falls clearly into one or other group. It is as if the potential of any one fibre became stabilized at about +35 mV, and then quite suddenly changed to −20 mV. No fibre was caught in the act of changing from one potential to the other and staying there, but several fibres were penetrated with the micro-electrode while they were oscillating between approximately these values. These oscillations were fairly slow

events, one cycle taking between 2 and 4 sec. The record in Fig. 6 is from the same experiment as Fig. 5. It was taken 108 min after the change of solution, and is fairly typical in its shape, though individual fibres varied somewhat in this respect. Only one fibre showing these oscillations was detected in this experiment. Between one and three oscillating fibres were

TABLE 5

Time in solution D, 100 mM-K, 220 mM-Cl (hr)	Time in solution F, 100 mM-K, 0 mM-Cl (hr)	$\frac{\text{dry wt.}}{\text{wet wt.}} \times 100$	$[K_i]$	$[Na_i]$	$[Cl_i]$ (m-mole/kg fibre water)
0	3	30·8	237	16	
		29·2			0
0	3	29·0	243	13	
		29·4			3
0	3	29·6	249	15	
		29·4			0
3	1	27·0	272	8	
		26·8			79
3	1	27·6	252	6	
		27·6			73
3	1	28·1	278	4	
		27·8			68
3	1	28·3	273	4	
		28·0			74
3	2	29·8	262	9	
		30·0			35
3	2	28·4	274	10	
		28·3			34
3	2	28·6	262	13	
		28·9			40
3	2	29·1	270	10	
		29·3			35
3	3	30·4	248	16	
		30·4			11
3	3	29·8	250	11	
		29·7			23
3	3	29·9	266	9	
		29·9			19
3	3	29·8	262	7	
		29·6			19

Brackets indicate muscles from the same frog.

found in each muscle that was examined under these conditions, and they were observed to last as long as about 4 min. If in the course of an hour every fibre passed through an oscillatory period of this length, in the experiment of Fig. 5 one would have expected to penetrate $3 \pm 1{\cdot}7$ oscillating fibres. The apparent rarity of finding fibres oscillating does not rule

out the possibility that every fibre does so during the transition from a positive to a negative internal potential.

Immediately after the transfer from the high-KCl solution (D) to the chloride-free solution (F), the membrane behaves as if it were much more

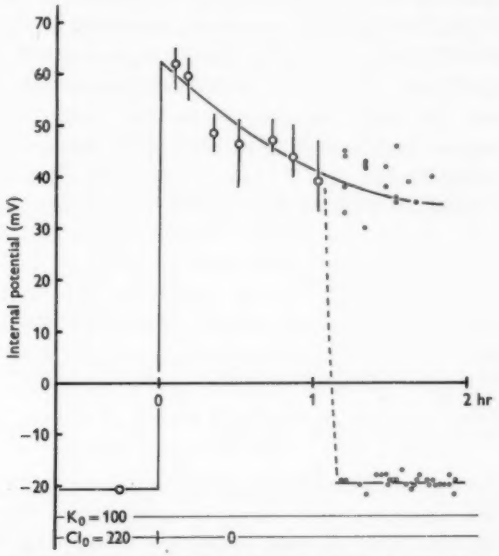

Fig. 5. The membrane potential of muscle fibres loaded with KCl in solution D (100 mM-K, 220 mM-Cl) and transferred to solution F (100 mM-K, 0 mM-Cl, sulphate solution). Large circles are the means of groups of 8–10 measurements, and the vertical lines join the highest and lowest measurement in each group. The small circles are individual measurements of membrane potential.

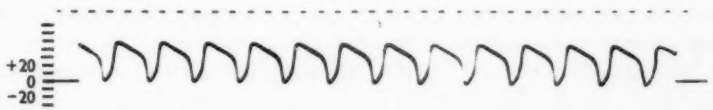

Fig. 6. Spontaneous oscillations of membrane potential. This record was taken from a fibre in the experiment illustrated in Fig. 5 108 min after the transfer from solution D to solution F. Calibration: voltage, 10 mV steps positive inside potential represented by an upward deflexion; time marker, seconds. The sharp steps at the beginning and end of the trace represent the penetration and withdrawal of the micro-electrode.

permeable to chloride than to potassium. As in the experiments where the external potassium concentration was reduced while keeping the chloride concentration constant, the low potassium permeability is associated with a large outward driving force on the potassium ion.

Ignoring the oscillations of membrane potential for the moment, the apparently sudden transition from about +40 to −20 mV could be explained by a sudden and sustained rise in the potassium permeability. If this were the case, the fibres with positive inside potentials should have a lower membrane conductance than those with negative internal potentials. The membrane conductance was therefore measured in the chloride-free sulphate solution (F) before and after the change in membrane potential. The potential produced by rectangular current pulses was measured with a micro-electrode at three distances from the current-passing micro-electrode. The space constant (λ) was determined from the steady levels of the electrotonic potential, and the time constant (τ_m) from the half-times at the different distances (Fatt & Katz, 1951; Hodgkin & Rushton, 1946).

TABLE 6

No. of fibres	Solution	Internal potential (mV, approx.)	λ (mm)	τ_m (msec)	r_m (Ωcm)	c_m (μF/cm)
9	Ringer's	−90	2·01	22·3	$12·7 \times 10^4$	0·18
3	Solution F (100 mM-K, 0 mM-Cl, sulphate)	+40	3·37	32·6	$28·7 \times 10^4$	0·11
3		−20	1·18	3·7	$3·0 \times 10^4$	0·12

Table 6 summarizes the results from six fibres. The results based on nine fibres in Ringer's solution are included for comparison. Since neither the diameter nor the internal specific resistance is easy to determine, the membrane resistance and capacity have not been calculated for a square centimetre of membrane but referred to one centimetre of fibre. While the figures for r_m and c_m are not strictly comparable between each group of fibres—both the diameter and internal specific resistance of each group are different because of volume and concentration differences—there appears to be a large decrease in membrane resistance associated with the change in membrane potential from +40 to −20 mV.

If the potassium permeability is inversely related to the magnitude of the outward driving force on the potassium ion, returning the membrane potential to the potassium equilibrium potential (−22 mV) by some means should increase the potassium permeability. If there was a group of fibres whose membrane potential did not oscillate, in which the potassium permeability depended only on the instantaneous value of the driving force and was independent of the time for which any particular driving force had been acting, there should be a period before the spontaneous transition when the membrane potential would be stable at either +40 or −20 mV. The possibility was tested in two ways; the membrane potential of fibres before the spontaneous transition (i.e. fibres with an internal potential of about +40 mV, which had been in the Cl-free sulphate solution (F) for

between 1 and 2 hr) was altered towards the potassium equilibrium potential either by raising the external chloride concentration to 220 mM, or by passing current through the membrane.

The external chloride concentration was altered suddenly by changing the fluid flowing past a muscle in a cell of the type described by Hodgkin & Horowicz (1959). Ideally such an experiment should be done with isolated fibres, to ensure a really rapid change of solution at the surface of the fibre. The presence of the other fibres of the intact muscle, lying alongside and below the impaled fibre, prevents the solution changing at the same rate over the whole surface. However, as long as the rates of change are not important, it is legitimate to use a whole muscle, and in one case (see below) a result depended on the fact that the external solution did not change rapidly. The experimental sequence was as follows; a muscle was loaded with KCl in the solution with extra potassium chloride (solution D; 100 mM-K, 220 mM-Cl), and then soaked in the chloride-free sulphate solution (solution F; 100 mM-K, 0 mM-Cl). With the muscle in this solution in the flow chamber a fibre was impaled, and with the micro-electrode still in the fibre, the solution flowing past the muscle was changed from the chloride-free (F) to the high-KCl solution (D) for a few seconds, and then back again to the chloride-free solution.

The shallow groove, in which the muscle was fixed and along which the solutions flowed, was earthed through an agar bridge of the chloride-containing solution. The change in potential of the fluid in the groove with respect to earth for the sequence of solution changes described above is shown in Fig. 7*a*. To obtain this record one cathode follower was connected to an electrode in the solution but outside a fibre, and the other cathode follower was earthed. With both cathode followers, recording differentially, connected to electrodes in the fluid no potential change was recorded when the solution was changed (Fig. 7*b*). However, with one electrode inside the fibre and one just outside a sudden change of potential of the same size as the junction potential is again recorded when the solution is changed, but if the reference cathode follower is earthed no sharp change in potential occurs. The most likely explanation of these observations is that the change of solution at the surface of the superficial fibres is not sufficiently rapid, and that for a short time there is a liquid junction of the two solutions between the external electrode and the surface of the impaled fibre. As soon as the solution immediately outside the impaled fibre has changed, the two micro-electrodes will record the true potential difference across the membrane (it is assumed that the potential at the tip of the external micro-electrode is unaltered). When the reference electrode is earthed, while there is no immediate potential step, a junction potential will develop and contribute to the measured membrane potential. The flow of solution past the muscle was made as fast as possible (about 5 ml./sec), but it was not sufficiently fast to eliminate the potential step recording with two micro-electrodes.

Figure 7 shows records from different fibres of the same muscle, taken after various periods in the chloride-free solution (F). Under these conditions the fibres are losing KCl, the internal concentration of chloride is falling but the concentration of potassium is more or less constant (see Table 5). About 30 fibres were tested in this way during the second hour

in the chloride-free sulphate solution, and none of them failed to return to a positive inside potential when the external chloride concentration was reduced from 220 to 0 mM. As the internal chloride concentration of the fibres fell, the rate at which the potential altered when the external chloride concentration was raised to 220 mM increased markedly. In the fourth record of Fig. 7, from a fibre which had been in the chloride-free

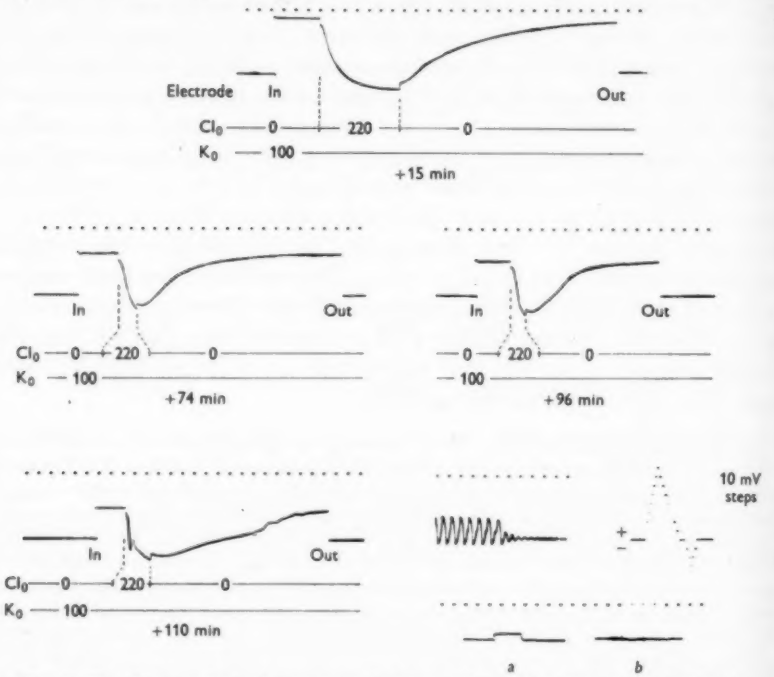

Fig. 7. The change of membrane potential caused by raising the external chloride concentration from 0 to 220 mM for short periods. The fibres had been loaded with KCl in solution D (100 mM-K, 220 mM-Cl) and transferred to solution F (100 mM-K, 0 mM-Cl) for the lengths of time indicated under each record. Calibration: voltage 10 mV steps, positive inside potential is represented by an upward deflexion; time marker, intervals of 3 sec.

solution for 110 min, the internal potential changed rapidly from +30 to −10 mV, then reversed to 0 mV before finally reaching −20 mV. This record suggests that as the internal potential falls the potassium permeability rises and moves the internal potential towards the potassium equilibrium potential (−22 mV) at a rate faster than it would move if the membrane potential depended on the instantaneous value of the chloride equilibrium potential. After about a second the potassium permeability appears to fall again, allowing the internal potential to move in a

positive direction towards the chloride equilibrium potential, which is itself moving slowly towards a value rather more negative than the potassium equilibrium potential as the external chloride concentration reaches 220 mM. Obtaining such a record depends to some extent on chance; the fibre must be in the right state, and the external chloride concentration must not rise too rapidly. Several other fibres showed the same behaviour, though the record in Fig. 7 was the most marked. The similarity between the shape of the spike on the record at 110 min and the oscillations taken from another fibre of the same muscle suggests that the same mechanism underlies both effects. Though it remains possible that no fibre was impaled during the period when it might have been capable of maintaining a stable internal potential of either $+40$ or -20 mV in the chloride-free solution, these results suggest that if the potassium permeability rises when the membrane potential is altered towards the potassium equilibrium potential, it falls again before the potassium equilibrium potential is reached.

The results of altering the membrane potential towards the potassium equilibrium potential by passing current through the membrane can also be interpreted in terms of a rise in the potassium permeability which is neither instantaneous nor maintained. The membrane potential of fibres loaded with KCl and losing these ions into the chloride-free sulphate solution (F) was altered by passing rectangular pulses of current through a micro-electrode inserted into the fibre. The potential was measured with a second micro-electrode inserted close by. Typical records are shown in Figs. 8 and 9. While the internal chloride concentration is still high, the potential change across the membrane is approximately symmetrical for small inward and outward currents. Large currents flowing inwards across the membrane cause a greater potential change than outward currents of the same strength, and the potential caused by large inward currents shows a tendency to decline even though the current remains constant. As the time that the muscle has been in the chloride-free solution increases and the internal chloride concentration falls, the potential produced by inward currents, which reduce the inside positivity, undergoes a series of changes. Long inward currents produce a large potential change initially but this is not maintained. For shorter currents the return of the potential to the resting level at the end of the current is delayed. This delay is not seen with long-lasting currents, but for both long and short currents there is a period after the end of the current when the inside potential becomes more positive than the resting level.

On two occasions the behaviour shown in records *A* and *B* of Fig. 9 was seen. With a short current pulse a 10 % increase in the strength of the current converted the delayed return to the resting level into a curve similar in shape to the oscillations previously described. A record from a

fibre with an oscillating membrane potential has been shown for comparison in Fig. 9*C* (the time scale of the oscillation is somewhat slower than in records *A* and *B*). By analogy with the mechanism of the action potential in physiological conditions the records of Fig. 9 suggest that there is a critical level of membrane potential between the resting level (+32 mV)

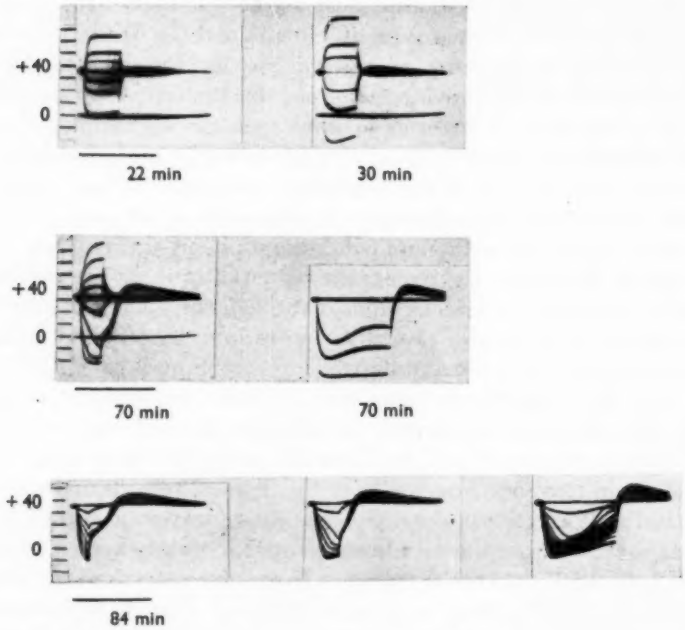

Fig. 8. The effect of rectangular current pulses on the membrane potential recorded close (about 300 μ) to the current-passing micro-electrode. The fibres were loaded with KCl in solution D (100 mM-K, 220 mM-Cl) and had been in solution F (100 mM-K, 0 mM-Cl) for the lengths of time indicated under each record. The records in the bottom line were all from the same fibre with different pulse lengths. Calibration: voltage, 10 mV steps, positive inside potential is represented by an upward deflexion; time, the horizontal line below the records represents 1 sec.

and the potassium equilibrium potential (−22 mV) at which the potassium permeability is sufficiently large for the outward potassium current to exceed the inward chloride current. When this occurs the potential moves towards the potassium equilibrium potential in the same way that the normal action potential moves toward the sodium equilibrium potential. The return of the potential towards the resting potential of +32 mV suggests that the potassium permeability falls, and this fall might be either spontaneous or initiated by a rise in chloride permeability. In the

absence of a change in the potassium or chloride permeability there would be no reason why the internal potential should not remain at -10 mV. The fact that the internal potential becomes more positive than the resting level before returning to it suggests that the potassium permeability falls to a value smaller than its resting value for a short period following the 'upside-down action potential'. The result of the proposed sequence of permeability changes would be a loss of both potassium and chloride from

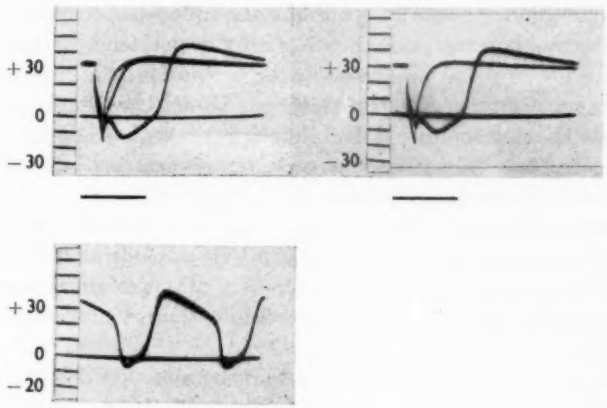

Fig. 9. Records *A* and *B* from two fibres 70 min after the transfer from solution D (100 mM-K, 220 mM-Cl) to solution F (100 mM-K, 0 mM-Cl). Rectangular pulses of current lasting 0·14 sec were passed through one micro-electrode and the potential was recorded through a second electrode close by. Current strength in arbitrary units; *A*, 20, 22·5, 25; *B*, 20, 25. Record *C*, spontaneous oscillations of membrane potential. Calibration: voltage, 10 mV steps, positive inside potential is represented by an upward deflexion; time, the horizontal line below each record represents 1 sec.

the inside of the fibre. In Fig. 9*C* the slow phase of potential change probably has the same explanation as the tail of increased positivity in *A* and *B*. The membrane potential oscillates because the time relations of the permeability changes prevent the existence of a value for the potential at which the net current can be zero for any length of time, and the membrane is alternately potassium- and chloride-permeable. Alternatively it would be possible for the chloride permeability to remain constant throughout and for the potassium permeability alone to alter. Once a fibre has started to oscillate potassium and chloride will continue to leave the fibre, but their movements will be separated in time. The internal chloride concentration will fall until it is no longer large enough to exert much effect on the membrane potential.

DISCUSSION

The experiments described in this paper support the conclusions which Hodgkin & Horowicz (1959) drew from measurements of membrane potential of isolated fibres when the external concentrations of potassium or chloride were altered suddenly. The present results suggest that the potassium permeability (P_K) varies with time as well as with the direction and size of the driving force on potassium, but they do not allow any detailed conclusions to be drawn about the chloride permeability (P_{Cl}). The results are, however, consistent with the variation of the chloride conductance (g_{Cl}) which would be predicted by the constant-field equations, assuming a constant chloride permeability. Under the conditions of these experiments the potassium permeability appears to be reduced when the internal electrochemical potential of potassium is greater than the external electrochemical potential. Hodgkin & Horowicz did not observe oscillations of membrane potential during the recovery in chloride-free sulphate solution of fibres loaded with extra KCl. It is possible that they did not insert a micro-electrode at the time when oscillations might have occurred, because a single fibre will not tolerate more than a limited number of penetrations.

Variations in the permeability will affect the rate at which a new steady state is approached when the external concentrations are altered, but they cannot affect the final steady-state concentrations. Boyle & Conway (1941) described the conditions which govern the concentrations of the permeable ions in the steady state. In their original formulation sodium was considered to be an impermeable ion, so that no energy was required to maintain the internal concentrations. Since the internal sodium is exchangeable with labelled external sodium and the fibre can under certain circumstances reduce its internal sodium concentration, the membrane cannot be impermeable to sodium. Conway (1957) has taken the sodium permeability into account and restated the theory, making allowance for the metabolic transport of sodium. He also uses the separate concepts of a 'steady state' and a 'balanced state'. Conway's 'steady state' is defined as a state in which the net movement of each ion is constant, and therefore a state in which the internal concentration may alter. In the 'balanced state' there is no net movement of any ion, so that there can be no alteration of the internal concentration. Conway's 'balanced state' corresponds to the definition of steady state used in this paper, i.e. one in which the internal concentrations do not alter with time.

When a muscle is in its normal environment the movement of sodium due to the concentration and potential gradients alone would cause the internal concentration to rise. If the internal sodium concentration is to

remain constant, this passive inward movement must be balanced by a metabolically driven outward movement, which could take the form of an outward current of sodium ions, or could be an electrically neutral process. Sodium could be pumped out with an anion, or the outward movement of sodium could be balanced by an active inward movement of potassium. Conway (1957) has considered the case where the sodium movement is not balanced by an active potassium movement or by an anion. In this case the membrane potential for the 'balanced state' (the state steady with respect to concentration) must be equal to the potassium equilibrium potential, in order that the potassium fluxes, which Conway considers to be entirely passive, shall be equal. This will also be true if the sodium is pumped out with an anion. While the exact value for the membrane potential *in vivo* is uncertain, the membrane potential of the isolated sartorius muscle is about 10 mV less than the potassium equilibrium potential when the external potassium concentration is 2·5 mM (Adrian, 1956). The membrane potential of isolated squid axons is certainly lower than the potential in the intact animal, but even there the potential appears to be lower than the potassium equilibrium potential (Hodgkin & Keynes, reported in Hodgkin, 1957). Conway considers that the departure of the membrane potential from the potassium equilibrium potential in the isolated muscle is due to the fact that the muscle is not strictly in the 'balanced state' and is gaining sodium and losing potassium.

It is, however, possible for a cell to exist in a state where the internal concentrations are not changing, and the membrane potential is not equal to the potassium equilibrium potential, provided at least part of the active efflux of sodium is balanced by an active uptake of potassium. Hodgkin (1957) has considered the case of a strictly neutral pump, in which one potassium ion is moved into the cell for each sodium ion moved out. It is assumed that the pump can keep pace with the entry of sodium and maintain a low internal sodium concentration, and that the chloride concentration ratio depends upon the membrane potential. In such a system the inside potential should be given by the equation

$$V = \frac{RT}{F} \ln \frac{[K_o] + \alpha[Na_o]}{[K_i] + \alpha[Na_i]}, \tag{1}$$

where α is the ratio of the sodium and potassium permeability, and the other symbols have their usual significance. The chloride concentration ratio will be given by the equation

$$\frac{[Cl_i]}{[Cl_o]} = \exp\left(\frac{VF}{RT}\right). \tag{2}$$

At external potassium concentrations in the physiological range the internal and external KCl products will not be equal, but provided α is small the

KCl products will approach equality as the external potassium concentration is raised. Hodgkin combined these equations with the equations used by Boyle & Conway to express the conditions of electro-neutrality and osmotic balance:

$$[K_i]+[Na_i]+[Cl_i]+\frac{A}{v} = C, \tag{3}$$

$$[K_i]+[Na_i]-[Cl_i]+\frac{zA}{v} = 0, \tag{4}$$

where C is the concentration of all particles in the external solution, v is the volume of the cell, A is the quantity of indiffusible particles inside the cell, and z is their average valence. The internal concentrations of potassium and chloride are given by the equations

$$0 = (1+z)[Cl_i]^2 + \{(\alpha-1)(1-z)[Na_i]-zC\}[Cl_i] - (1-z)\{[K_o]+\alpha[Na_o]\}[Cl_o], \tag{5}$$

$$[K_i] = \frac{(1+z)[Cl_i]-(1-z)[Na_i]-zC}{1-z}. \tag{6}$$

The volume of the cell relative to that in Ringer's solution can be obtained by inserting the calculated values for $[K_i]$ and $[Cl_i]$ for any given external concentrations into equation (3).

It is worth while comparing the values predicted by equations (5) and (6) with the experimental equilibrium values in solutions B, D, and F (Table 1). The ratio of the permeabilities of sodium and potassium was taken as 0·01:1 (Hodgkin & Horowicz, 1959), and it was assumed that the pump was able to keep the internal sodium concentration at 15 mM in these solutions. The concentration and average valence of the internal indiffusible particles was obtained by inserting into equations (3) and (4) the experimentally determined internal concentrations of potassium and chloride for a muscle in Ringer's solution. Table 7 gives the calculated and observed values for the three solutions in which the steady-state concentrations were measured. The agreement is satisfactory, and confirms, if confirmation is needed, that the kind of mechanism proposed by Boyle and Conway describes the distribution of potassium and chloride very adequately. Since the external potassium concentration in these solutions was high, the agreement between the experimental and predicted values does not constitute evidence for a Na-K coupled pump, but it is obvious that a coupled pump is not ruled out. The best evidence for the coupling of the active movements of sodium and potassium is from human red cells (Glynn, 1956, 1957). There is also evidence from the axons of the squid and cuttlefish (Hodgkin & Keynes, 1955; Hodgkin, 1957) and from frog sartorius muscle (Keynes, 1954). It is not certain in any of these cases

whether the coupling is always one-for-one, but as long as there is any inward metabolic movement of potassium, the cell will be able to maintain a constant internal ionic composition even though its membrane potential differs from the potassium equilibrium potential.

The rate at which any new steady state is approached when the external conditions are changed will depend on the permeabilities of the substances which must either enter or leave the fibre. When the external concentration of potassium or chloride is changed, the membrane potential will immediately take up a value which produces equal net movements of these

TABLE 7

	External concentration (m-equiv/l.)		Internal concentration (m-mole/kg fibre water)					Fibre volume (% of vol. in Ringer's soln.)	
			Na^+	K^+		Cl^-			
Solution	K^+	Cl^-	Meas.	Meas.	Calc.	Meas.	Calc.	Meas.	Calc.
Ringer's	2·5	120	—	139	—	3·8	3·1	—	—
B	50	170	13	192	188	47	46	90	96
D	100	220	9	242	238	97	93	89	93
F	100	0	15	243	261	0	0	58	54

The mean valence of the internal anion was calculated by equations (3) and (4); $z = -1{\cdot}63$. The internal concentrations in the other solutions were calculated by equations (5) and (6). $[Na_i]$ was assumed to be 15 m-mole/kg fibre water in all solutions. The values for $[K_i]$ and $[Cl_i]$ in Ringer's solution were taken from Adrian (1956, and unpublished). The concentration of all particles in Ringer's solution (C) was taken to be 250 mg ions/l.

two ions (i.e. the net current will be zero; any contribution of Na to the net current is ignored in the following discussion). The constant-field equations (7) and (8) can be used to calculate P_K and P_{Cl} from the net movements if the immediate change in membrane potential is known (Hodgkin & Horowicz, 1959). The net inward movement of chloride (M^i_{Cl}) is given by the equation,

$$M^i_{Cl} = P_{Cl}\frac{VF}{RT}\left\{\frac{[Cl_o]-[Cl_i]\exp(-VF/RT)}{1-\exp(-VF/RT)}\right\}. \quad (7)$$

The net outward movement of potassium (M^o_K) is given by the equation,

$$M^o_K = P_K\frac{VF}{RT}\left\{\frac{[K_i]-[K_o]\exp(-VF/RT)}{1-\exp(-VF/RT)}\right\}. \quad (8)$$

In the experiments of this paper the instantaneous change in membrane potential could not be measured; it was either too small, or the change of potential after the initial change was too rapid. However, Hodgkin & Horowicz (1959) have shown that P_{Cl} is constant under a wide variety of conditions, and this fact can be used to get a rough estimate of P_K. If P_{Cl} and the initial net movement of chloride are known, equation (7) will give the membrane potential immediately after the change of external

conditions. Ignoring any movement of sodium, the movement of potassium will be equal to the movement of chloride, so that equation (8) can be solved for P_K by using the value of V calculated by equation (7). When the external potassium concentration, but not the external chloride concentration, is altered, the initial change in membrane potential is very small and P_K will not be seriously in error if the membrane potential before rather than after the change of solution is used in equation (8).

An estimate of the initial rate of chloride loss can be made from the slow rate of change in potential in the experiments of Figs. 3 and 4, where the external potassium concentration was reduced to 2·5 mM. The assumption is that during the slow change the membrane potential does not differ from the chloride equilibrium potential by more than a fraction of 1 mV. The net movement of chloride was estimated by calculating $[Cl_i]$ from the potential at two times and assuming that the fibres had a diameter of 80 μ throughout. This procedure was preferred to the direct analysis, because it is possible that the rate of loss from the deep and superficial fibres is not equal (see p. 166). When the external potassium concentration is reduced from 50 to 2·5 mM (Fig. 3) the net loss of chloride is 6 pmole/cm²/sec, and when it is reduced from 100 to 2·5 mM (Fig. 4) the net loss is 1 pmole/cm²/sec. Equation (7) predicts an initial potential change of less than 1 mV for both cases if P_{Cl} is equal to 4×10^{-6} cm/sec (Hodgkin & Horowicz, 1959). In neither case was there a detectable change in potential when the external potassium concentration was altered. In the first experiment (Fig. 3) the outward driving force on the potassium ion ($V-V_K$) was 76 mV and P_K was equal to $0{\cdot}06 \times 10^{-6}$ cm/sec, and in the second experiment (Fig. 4) the driving force was 93 mV and P_K was $0{\cdot}01 \times 10^{-6}$ cm/sec. If there is an inward movement of sodium, the movement of potassium will exceed the movement of chloride and P_K will have been underestimated. Hodgkin & Horowicz (1959) estimated that P_K is $0{\cdot}04 \times 10^{-6}$ cm/sec when ($V-V_K$) is 93 mV.

Equations (7) and (8) can be used to give a roughly quantitative explanation of the observations which were made on fibres losing KCl into the chloride-free sulphate solution (Fig. 5; solution F, 100 mM-K). The net movement of chloride is given by equation (7). In Fig. 10*A* the calculated net movement of chloride ($P_{Cl} = 4 \times 10^{-6}$ cm/sec) is plotted against the internal potential for the conditions which apply when a fibre, loaded with KCl, is transferred to the chloride-free sulphate solution. Since there is no external chloride, there can be no inward movement, and since all the fibres start with an internal chloride concentration of 100 m-mole/kg fibre water they will start with the chloride movement versus potential relationship given by the top member of the family of curves in Fig. 10*A*. As the fibres lose their internal chloride, the curve will be scaled down in

proportion to the internal concentration, and when all the chloride has been lost there can be no chloride movement in either direction. Similarly the net movement of potassium is given by equation (8). In this case, however, P_K is not constant, but depends on the driving force, and the

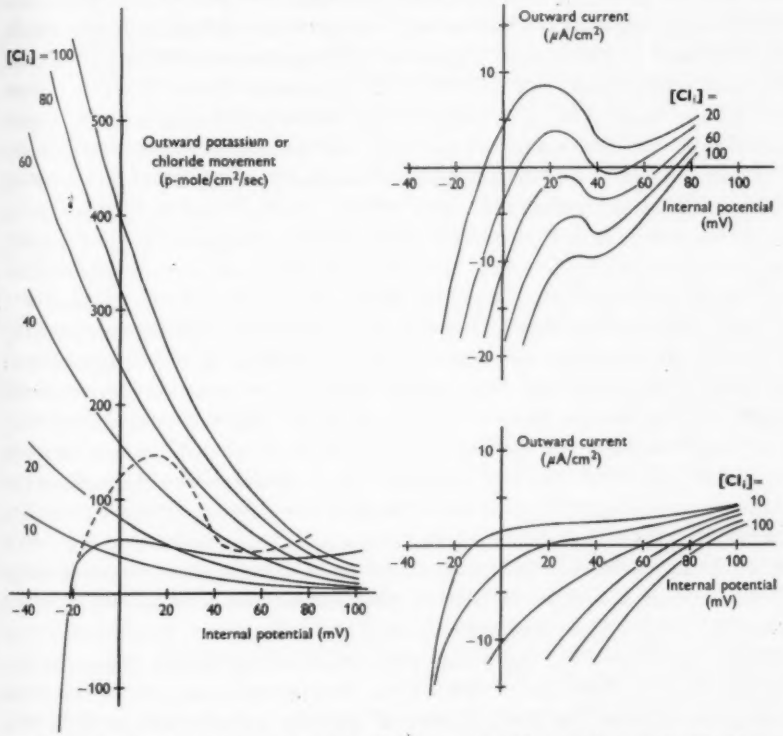

Fig. 10. *A*, theoretical curves for the movement of potassium and chloride, calculated from the constant-field equations (7 and 8) and the values of P_K given by Hodgkin & Horowicz (1959) for muscles loaded with KCl in solution D (100 mM-K, 220 mM-Cl) and losing potassium and chloride into solution F (100 mM-K, 0 mM-Cl). Abscissa, internal potential in mV. Ordinate, outward movement in pmole/cm²/sec. *B* and *C*, curves of membrane current against membrane voltage derived from *A*. Abscissa, internal potential in mV; ordinate, outward current in μA/cm². For assumptions and explanation see text.

internal potassium concentration remains constant because the fibre shrinks. A single curve, therefore, describes the relation between the movement of potassium and the internal potential. The solid line which crosses the family of chloride curves in Fig. 10*A* was calculated from equation (8) combined with the data of Hodgkin & Horowicz for P_K versus $(V - V_K)$. P_K varies between $0{\cdot}04 \times 10^{-6}$ and $7{\cdot}0 \times 10^{-6}$ cm/sec, and at the potassium

equilibrium potential (− 22 mV) P_K was taken as equal to $1{\cdot}4 \times 10^{-6}$ cm/sec (Table 9, Hodgkin & Horowicz, 1959).

If it is assumed that no other ions contribute to the total membrane current, the current–voltage relation of the membrane at any particular time will be given by the difference between the potassium movement curve and the chloride movement curve appropriate to the internal chloride concentration at that time. Figure 10*B* shows the set of current–voltage relations which would apply if the solid potassium line of Fig. 10*A* were true at all times. The membrane would show rectification in the same direction as that first described by Katz (1949) for muscle fibres in isotonic potassium sulphate, and in the opposite direction to the delayed rectification of the squid axon (Hodgkin, Huxley & Katz, 1949). There would, however, be no discontinuity in the repolarization which takes place in the chloride-free sulphate solution (see Fig. 5) as the fibre loses potassium and chloride.

If the relation between the potassium permeability and the driving force on the potassium ion was rather less steep, and the limiting value of P_K was rather greater than the figures of Hodgkin & Horowicz suggest, then the dotted curve in Fig. 10*A* would describe the relation between the potential and the net movement of potassium. As the outward driving force was increased the outward movement of potassium would increase to a maximum, but a further increase in driving force would decrease the outward movement. Figure 10*C* shows the current–voltage curves for the membrane if the dotted curve for potassium movement in Fig. 10*A* were true at all times. Such a set of curves with regions of negative slope would explain a sudden transition from a positive to a negative inside potential as the fibres lost chloride and potassium into the chloride-free sulphate solution (F). Agreement in detail could hardly be expected, because the contribution of other ions, for instance sodium, to the total membrane current has been neglected, and the dotted curve in Fig. 10*A* was rather arbitrarily drawn.

Neither set of current–voltage curves alone could account for the oscillatory behaviour of the membrane potential during the transition period. However, if the dotted curve of potassium movement applies when the inside of the fibre has been positive for a time, and the solid potassium movement curve applies when the inside potential has been at or near the potassium equilibrium potential for a time, then conditions will occur when both the oscillations and the 'upside-down action potential' would be expected. In terms of permeability it is suggested that when the internal potential is moved in a positive direction from the potassium potential, the potassium permeability falls to a minimum but then rises to a value rather greater than the minimum. When the internal potential is moved in a negative direction towards the potassium equilibrium potential, the

potassium permeability rises but then falls to a value rather less than that to which it has just risen. The differences in the behaviour of the membrane potential at different times after the transfer of the KCl-loaded muscle to the chloride-free sulphate solution depend on the progressive fall in the internal chloride concentration, and it is unnecessary to postulate any changes in the chloride permeability. In the absence of precise information about the potassium permeability changes, it is impossible to predict the shape of the oscillations or of the 'upside-down action potential'. Nor does it seem worth while to pursue these rather incidental and unphysiological findings much further, although the analogy with the normal action potential is interesting and suggests a large number of questions which remain unanswered. Is the 'upside-down action potential' propagated? Is the height of the 'overshoot' dependent on the external potassium concentration?

Under the conditions of these experiments the pathway for the passage of potassium ions across the muscle membrane has the properties of a rectifier, and the resistance to the movement of potassium ions is high when there is a maintained outward movement. This effect, which has been called anomalous rectification, is in the opposite direction to delayed rectification and to the rectification predicted by the constant-field equations. If delayed rectification accounts for the rapid repolarization of the muscle action potential, as it does in the squid action potential, then one must suppose that when the fibre is depolarized delayed rectification precedes anomalous rectification, or that when the membrane is depolarized from its resting level of -92 mV only delayed rectification occurs. A third possibility is that there are two types of potassium channel, with opposite rectifier properties, in parallel with each other. It is tempting to suggest that the potassium permeability is high during the fast repolarization of the action potential, but that it falls at the start of the negative after-potential when the repolarization becomes slow. The negative after-potential would then represent the movement of some other ion, perhaps chloride, carrying the charge necessary to repolarize the membrane fully. The experiments reported in this paper give no evidence on this point, because they describe the behaviour of muscles whose potassium permeability has been reduced for considerable periods.

SUMMARY

1. When the KCl concentration of the solution surrounding a frog sartorius muscle is raised, $[K_i]$ and $[Cl_i]$ at equilibrium agree with the values predicted by a Donnan system of the Boyle–Conway type.

2. When KCl must move across the membrane to reach a new equilibrium, inward movement of KCl is rapid, but outward movement is slow.

The ionic movements were followed by chemical estimation of the internal concentrations.

3. Muscles loaded with KCl in high-KCl solutions repolarize very slowly when returned to solutions with a normal potassium concentration. If the external potassium concentration is reduced to the normal value (2·5 mM) in a series of steps repolarization is more rapid.

4. Muscles loaded with KCl in a solution containing 100 m-mole K/l. have an internal potential of +60 mV initially when the external chloride is replaced by sulphate. The internal potential falls to about +40 mV as K and Cl are lost, and then the potential of most, if not all, fibres oscillates spontaneously between about +40 and −20 mV for a period of a few minutes, after which the internal potential becomes stable at −20 mV. In a very small number of fibres with a positive internal potential a short inward pulse of current evoked an 'upside-down action potential' resembling a single cycle of the oscillations.

5. Fibres in the Cl-free solution with internal potentials of +40 mV have a membrane resistance about ten times that of fibres in the same solution with an internal potential of −20 mV, and about twice that of fibres in Ringer's solution.

6. These observations may be explained by supposing that the potassium permeability falls when there is an outward movement of potassium and that it is related inversely to the size of the outward driving force on potassium. The oscillations of membrane potential and the 'upside-down action potential' imply that the potassium permeability is not dependent on the driving force alone but changes both with driving force and time.

I am greatly indebted to Professor A. L. Hodgkin and Dr P. Horowicz for very helpful discussion throughout the course of this work. The expenses of the work were met by grants from the Rockefeller and Nuffield Foundations.

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THE RELATIONSHIP OF THE OXYGEN CONSUMPTION TO THE CONTRACTION OF THE CAT PAPILLARY MUSCLE

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Most studies of the relationship between the oxygen consumption and the work of the heart have been performed *in vivo* or with isolated but intact heart preparations; and relatively few investigators have used isolated strips of cardiac muscle (Starling & Evans, 1914; Evans & Matsuoka, 1915; Gollwitzer-Meier, Kramer & Krüger, 1936; Lee, 1954). Since the influence of haemodynamic changes can be completely eliminated in studies with isolated muscle strips, the information obtained with such preparations is useful for an understanding of energy–work relationship in the cardiac muscle itself. Previously the author has reported on studies of this type, utilizing a manometric method which allowed the simultaneous measurement of oxygen consumption and of contraction of the papillary muscle from cat heart (Lee, 1953). Although this method was satisfactory for certain purposes, it had some disadvantages, the major one being its limited sensitivity to changes of oxygen concentration. Since in such investigations extremely small and thin muscles must be used to insure an adequate oxygen diffusion throughout the tissue, the sensitivity of the device for measuring oxygen concentration has to be extremely high. The present paper describes the experiments in which a method of adequate sensitivity based on a polarographic device for the measurement of the oxygen concentration in the medium was utilized to study the relationship of the oxygen consumption to the contraction of the cat papillary muscle under various experimental conditions. In addition, the newly designed apparatus for this work also permitted variation of tension on the muscle during the course of a single experiment. Special attention was focused upon the influence of tension and the frequency of contraction on the oxygen consumption and contractility of the papillary muscle.

METHODS

The principle of the polarographic device used by Carlson, Brink & Bronk (1950) for their studies on the oxygen consumption of nerve was applied to measuring the oxygen concentration of the medium. The system consisted of a muscle chamber, recording systems for electrode current and contraction, stimulating apparatus and a syringe driver device.

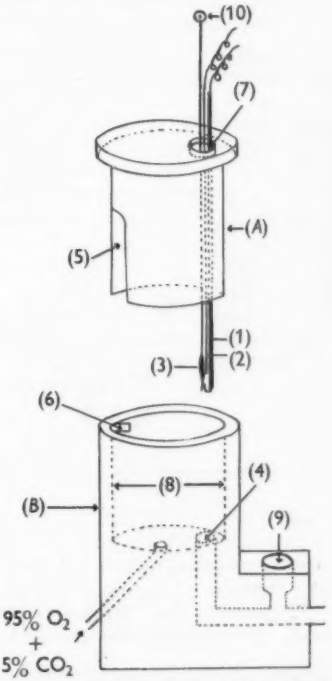

Fig. 1. Diagram of the muscle chamber. *A*, muscle holder; *B*, perfusion reservoir; 1 and 2, stimulating electrode; 3, muscle; 4, capillary channel, diameter 2·5 mm; 5, guide rail; 6, guide projection; 7, orifice for stimulating electrodes, diameter 6 mm; 8, fluid bath, diameter 3·9 cm; 9, orifice for oxygen electrode holder, diameter, 7 mm; 10, RCA transducer tube 5734.

The muscle chamber was made of lucite and consisted of a muscle holder and a perfusion-fluid reservoir. Figure 1 is a diagrammatic outline of the chamber. At the top of the muscle holder (*A*) was a 6 mm diameter opening through which stimulating electrodes and a thread from one end of muscle were passed. The stimulating electrodes (1 and 2) were made of platinum insulated with polyethylene tubing. The papillary muscle (3) was tied to one electrode of the muscle holder and was guided into a 2·5 mm diameter capillary channel (4) of the fluid reservoir (*B*), which opened at the bottom of the latter as shown in Fig. 1. A plastic plug containing an oxygen electrode was inserted into a 7 mm diameter opening (9) to the capillary channel distal to the muscle.

The arrangement of the entire system is shown in Fig. 2. The electrode-current-recording system consisted of a Northrup Speedomax galvanometer with chopper amplifier (*G*),

a 1·5 V battery (*BE*), a calomel half-cell (*D*) and an oxygen electrode (*E*) in circuit. The oxygen cathode was made of a platinum wire (0·005 in., 0·127 mm diam.) and a supporting glass rod (2 mm outside diam.). The platinum wire was sealed into a glass tube and then the sealed end of the glass tube was carefully ground on a plastic block to expose a clear round cross-section of the platinum wire. The tip of the electrode was covered with a polyethylene membrane tied with a fine silk thread. This electrode was inserted through the centre hole in the plastic electrode holder and fixed in the proper position with de Khotinsky cement.

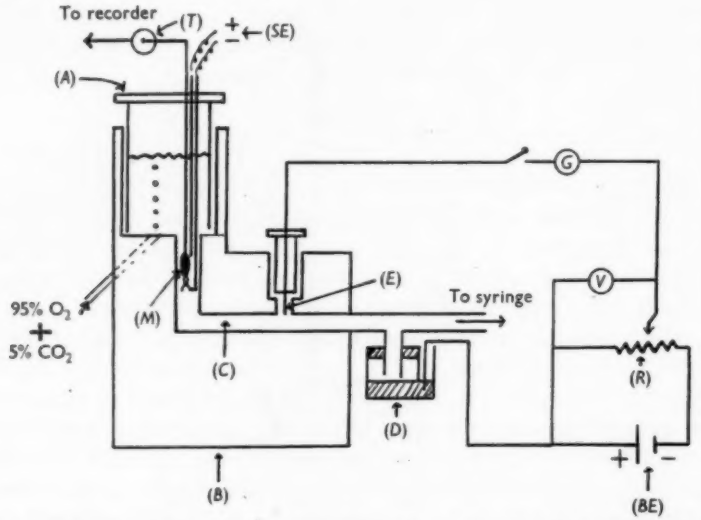

Fig. 2. Diagram of the entire recording system. *A*, muscle holder. *B*, perfusion fluid reservoir. *BE*, 1·5 V battery. *C*, capillary channel. *D*, calomel half cell. *E*, oxygen electrode. *G*, galvanometer. *M*, papillary muscle. *R*, rheostat. *SE*, stimulating electrodes. *T*, RCA transducer tube 5734. *V*, voltmeter.

After a muscle was placed in the capillary channel the thread from the muscle was connected to a spring which was in contact with an RCA transducer tube 5734 (T). Muscles were stimulated by a Grass Stimulator (Model S4) and the development of the isometric contractile tension of muscles was recorded on a Sanborn Twin Viso through the RCA tube. An extremely accurate constant-flow rate syringe driver (Baltimore Instrument Co.) which met the specifications required for the present study, pulled the perfusion fluid from the reservoir through the capillary channel (*C*) past the muscle, as indicated by the arrow, at a constant rate of 0·9966 ml./hr or 1·892 ml./hr. This rate of flow was selected so that the maximum extraction of oxygen by the muscle from the perfusing fluid would not exceed more than 25% of total oxygen present in the solution, thus simulating the physiological condition of oxygen extraction. A gas mixture of O_2 95 + CO_2 5% was continuously bubbled through the reservoir. The distance between the closer end of the muscle and the oxygen electrode was 1·2 cm. The distance between the tissue and the oxygen electrode, which is required for a uniform oxygen tension across the stream at the electrode in the solution, was calculated according to the formula given by Carlson *et al.* (1950) and found to be 0·37 cm under the present conditions. Therefore the distance between the muscle and electrode in this case was more than adequate to insure a homogeneous concentration of

oxygen across the stream at the position of the oxygen cathode. When the oxygen cathode was made several tenths of a volt negative with respect to the calomel half-cell, by adjusting the rheostat (*R*) in the presence of a battery (*BE*), oxygen molecules around the oxygen cathode were electrolytically reduced, the rate of reduction of oxygen being proportional to the concentrations of oxygen. The current flowing through the oxygen cathode was recorded on the galvanometer (*G*) and measured the oxygen concentrations in the solution at the electrode after it had been pulled past the muscle at a constant flow rate. In this method it is necessary to calibrate the individual electrode for different concentrations of oxygen before use. With all electrodes used in this work the electrode current measured by the galvanometer was linearly proportional to the oxygen concentration present in the solution. The theoretical principles basic to this polarographic method have been described previously (Connelly, Bronk & Brink, 1953).

All experiments were carried out at 37° C, in Krebs–Ringer solution with bicarbonate buffer (Umbright, Burris & Stauffer, 1957). To maintain this temperature the muscle chamber was placed in the constant-temperature box made of lucite. All Q_{O_2} values in this study were calculated on a wet weight basis. Since the perfusion fluid had to travel the distance between the muscle to the oxygen electrode, there was a time lag between the actual change of oxygen concentration at the muscle and its reflexion at the oxygen electrode. This time lag was found to be 7·5 min with the flow rate of 0·9966 ml./hr. In all the experiments a correction was made for this lag in order to obtain the actual temporal relationship between the oxygen consumption and the contraction of muscle. Muscles having diameters ranging from 0·3 to 0·6 mm were selected in order to insure the adequate oxygenation of the whole muscle, since it had been found that muscles having a diameter larger than 1 mm were inadequately oxygenated at the centre of the muscle (Lee, 1953).

RESULTS

Resting respiration of papillary muscle

The oxygen consumption of a muscle in the resting state without tension was studied in twenty-four experiments. The weight of the wet muscles ranged from 1·5 to 4·5 mg and the average Q_{O_2} on a wet-weight basis was found to be 1·16, with a standard error of 0·28. This Q_{O_2} of resting muscle without tension will be referred to as a 'basal resting Q_{O_2}'.

Effect of tension of resting muscle on the oxygen consumption

The application of tension to resting muscle was accompanied by an increase in Q_{O_2}. This increment of Q_{O_2} of resting muscles due to increase in resting tension will be referred to as a 'tension Q_{O_2}'. Figure 3 illustrates the relationship between the tension applied to resting muscle and the oxygen consumption of the muscle in a typical experiment. Over a range of muscle tension from 0 to 12 g/mm² of muscle, the Q_{O_2} increased progressively as the tension increased. A further increase in muscle tension beyond 12 g/mm², however, was accompanied by an insignificant change in the oxygen consumption. Table 1 summarizes the results obtained at three different tensions. The average maximal increase in the Q_{O_2} of resting muscle due to increased tension was 93 % ± 7·9 (S.E.) of the 'basal resting Q_{O_2}', and the average minimum tension required for this maximal increase in Q_{O_2} was 13 g/mm² ± 2·5 (S.E.).

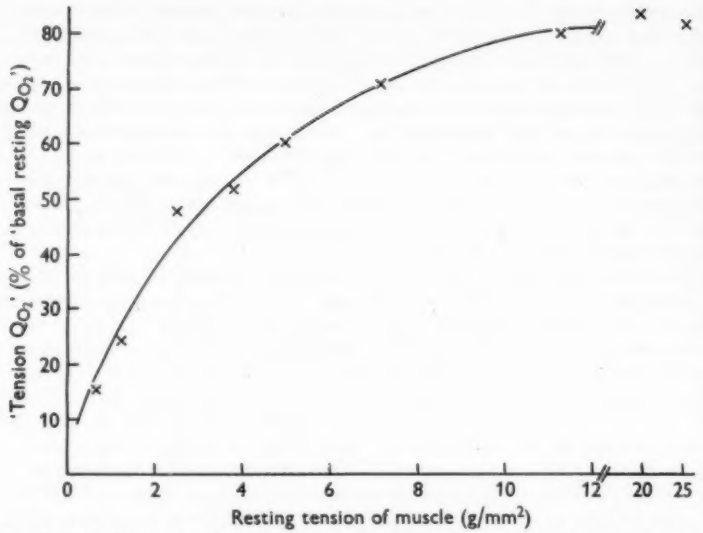

Fig. 3. The effect of resting tension on oxygen consumption of non-contracting muscle. Muscle diameter 0·37 mm; wet weight 2·8 mg.

TABLE 1. Effects of resting tension on oxygen consumption

No. of expts.	Tension (g/mm²)	Mean 'tension Q_{O_2}' (% of 'basal resting Q_{O_2}' ± S.E.)
7	2	32 ± 6·1
9	6	66 ± 8·3
7	12	89 ± 7·9

Changes in oxygen uptake by muscle during contraction

When the muscle was stimulated electrically at a constant frequency under a given tension, the rate of oxygen consumption increased to a new constant value. When the stimulation was terminated the rate of oxygen uptake gradually subsided to the previous resting level. A tracing of a typical record of the effect of a period of stimulation on electrode current is presented in Fig. 4. The decrease in current during the period of stimulation measured the increase in the amount of oxygen removed by the muscle from the passing medium. Since the flow rate and calibration constant were both unvarying, the change in the current was proportional to the change in the rate of extraction of oxygen from the solution. The difference in electrode current between the initial and final steady values measured the change in rate of oxygen consumption caused by the contraction of the muscle. This increment of Q_{O_2} due to contraction will be

referred to as 'activity Q_{O_2}'. As is seen in Fig. 4, during the isometric contraction of a papillary muscle initiated by electrical stimulation at a rate of 60/min, the increment of tension due to isometric contraction (contractile tension) reached a plateau in a few minutes where it remained relatively steady over many hours. Both the transition in the rate of oxygen consumption on going from the resting state to a state of rhythmic contraction, and the transition on returning to the resting rate, are described by exponential functions of time, except for the initial phase of each transition; but the time constant of the latter transition is longer than that of the former.

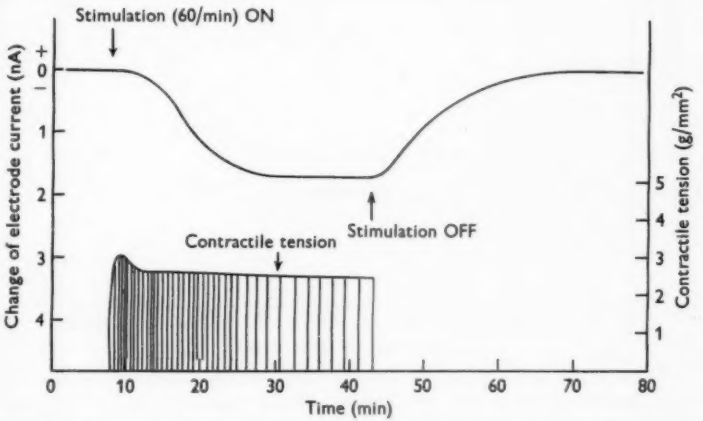

Fig. 4. The change in electrode current and contractile tension before, during and after electrical stimulation of the papillary muscle. Muscle diameter 0·32 mm; wet weight 3·8 mg; tension 2 g/mm².

Effect of the frequency of stimulation on the 'activity Q_{O_2}' and contractility

In these experiments the tension of the resting muscle was kept constant and the frequency of stimulation was varied over a range of 10–150/min. Both the increment in the oxygen consumption due to contraction ('activity Q_{O_2}') under a given resting tension, and the contractile tension developed, were dependent on the frequency of contraction. Results obtained in a typical experiment are shown in Fig. 5. It should be noted that at lower frequencies of stimulation there is a rise in 'activity Q_{O_2}' as the frequency of stimulation is increased. However, at a rate of 110/min or greater further increase in the rate of stimulation did not result in a significant increase in the oxygen consumption. Initially the increase in the frequency of contraction was accompanied by an increment in the contractile tension. However, a further increase in the rate of stimulation

above 70/min (in the expt. in Fig. 5), was accompanied by a reduction in the contractile tension. The mean values of contractile tension and 'activity Q_{O_2}' for different frequencies are given in Table 2.

The 'activity oxygen consumption per contraction' was calculated by dividing 'activity Q_{O_2}' by frequency of contraction per hour, and the

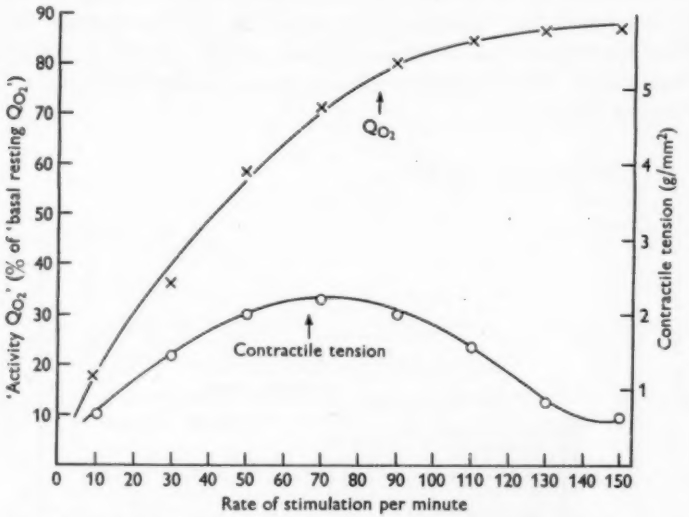
'Activity Q_{O_2}' (% of 'basal resting Q_{O_2}')
Contractile tension (g/mm²)
Q_{O_2}
Contractile tension
Rate of stimulation per minute
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
10 20 30 40 50 60 70 80 90
1 2 3 4 5

Fig. 5. The effect of frequency of stimulation on oxygen consumption and contractile tension developed by the muscle. Muscle diameter, 0·41 mm; tension, 4 g/mm²; wet weight, 4 mg.

TABLE 2. Effects of frequency of contraction on oxygen consumption and contractile tension

No. of expts.	Rate of contraction (per minute)	'Activity Q_{O_2}' (% of 'basal resting Q_{O_2}' ±S.E.)	Contractile tension (g/mm², ±S.E.)
6	30	39±8	1·3±0·16
5	70	73±11	2·8±0·32
5	110	91±16	1·5±0·28

Resting tension of muscle ranged between 3 and 6 g/mm².

values for different frequencies are given in Table 3. This table makes it clear that there is an inverse relationship between the frequency and the 'activity oxygen consumption per contraction'.

The ratio of isometric contractile tension : 'activity oxygen consumption per contraction', was calculated for each frequency of stimulation and was assumed (for reasons discussed later, p. 197) to represent an index of the mechanical efficiency of the muscle. A plot of this ratio against frequency

in a typical experiment is shown in Fig. 6. In this experiment efficiency rose to a maximum at around 90/min and then declined at higher frequencies.

TABLE 3. Activity oxygen consumption per contraction

Rate of contraction (per min)	10	30	60	90	120	150
Q_{O_2} per contraction ($\times 10^{-4}$ ±S.E.)	3·25±0·36	2·78±0·22	2·38±0·19	1·83±0·09	1·27±0·12	1·03±0·14

The oxygen consumption per contraction = $\frac{Q_{O_2} \text{ due to contraction}}{\text{rate of contraction per hour}}$; resting tension of muscle ranged between 4·1 and 6·6 g/mm².

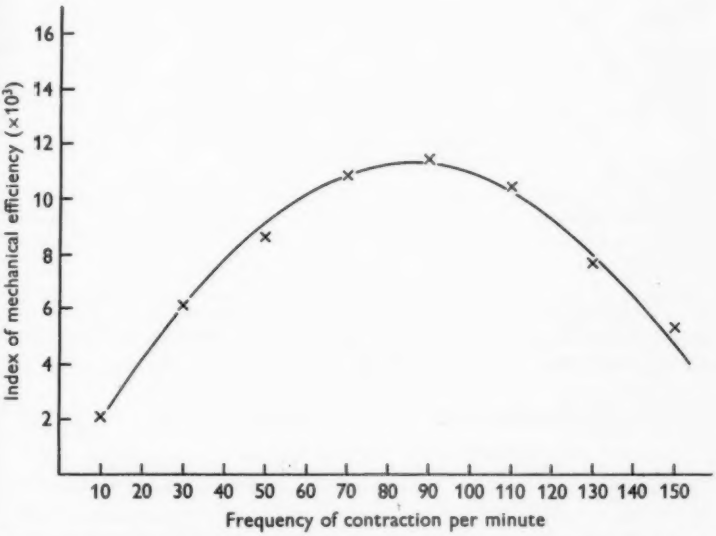

Fig. 6. The effect of frequency of contraction on the mechanical efficiency of the papillary muscle. Muscle diameter, 0·5 mm; wet weight, 7 mg; tension, 4 g/mm².

Effect of resting tension on the 'activity Q_{O_2}' and contractile tension of muscle being stimulated at a constant frequency

In muscles stimulated at a rate of 60/min, it was found that both the contractile tension and 'activity Q_{O_2}' varied with the resting tension. As is shown in Fig. 7, at low to moderate resting tension the 'activity Q_{O_2}' increased as the resting tension was increased. However, a further increase of the resting tension beyond approximately 12 g/mm², led to a slight decrease in the 'activity Q_{O_2}'. An increase in the contractile tension also occurred as the resting tension of the muscle was increased up to

approximately 6 g/mm². However, a further increase in the resting tension (beyond 6 g/mm²) was accompanied by a considerable decline in the contractile tension. This decline in contractile tension beyond a certain resting tension is in accord with Starling's law (1915). A summary of the results concerning the effect of resting tension on the 'activity Q_{O_2}' and on the contractile tension is given in Table 4.

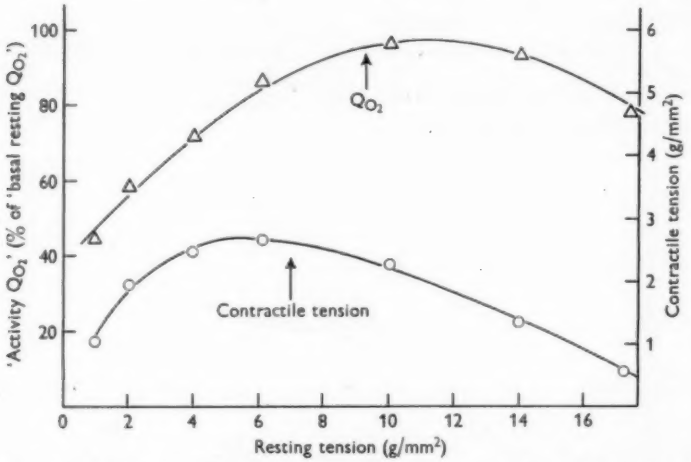

Fig. 7. The effect of resting tension on the activity respiration and tension developed by the muscle during contraction. Muscle diameter, 0·43 mm; wet weight, 3·2 mg. Stimulation frequency, 60/min.

TABLE 4. Effects of resting tension on oxygen consumption and contractile tension

No. of expts.	Resting tension (g/mm²)	'Activity Q_{O_2}' (% of 'basal resting respiration', ±S.E.)	Contractile tension (g/mm², ±S.E.)
6	1	39±9	1·0±0·21
5	4	66±8·6	2·42±0·35
6	10	99±11·2	2·1±0·33

The ratio, isometric contractile tension : 'activity oxygen consumption per contraction', at various resting tensions was calculated as described previously and taken as a hypothetical index representing the mechanical efficiency of these resting tensions. In Fig. 8 this index is plotted against the tension of the resting muscle. In the experiment illustrated, as well as in other similar experiments, efficiency rose sharply with resting tension, reached a maximum at about 3 g/mm² and then declined with further increase in tension.

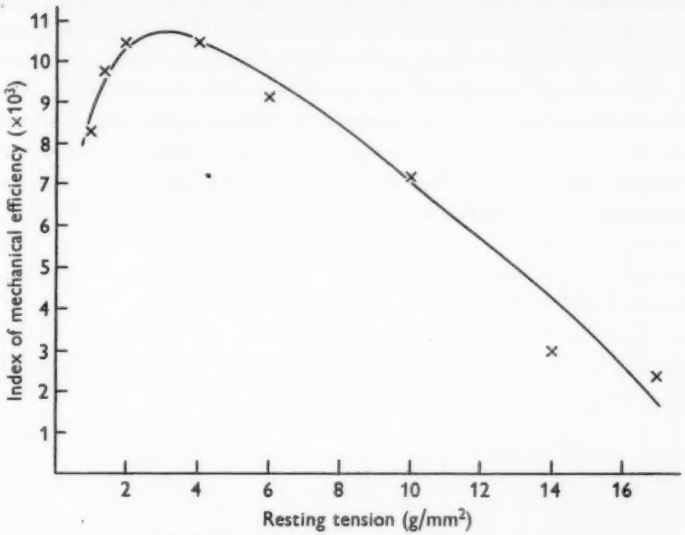

Fig. 8. The effect of resting tension on the mechanical efficiency of the papillary muscle. Muscle diameter, 0·35 mm; wet weight, 4·1 mg; stimulation frequency 60/min.

The relationship between resting tension, total oxygen consumption and length of muscles contracting at a constant rate

The contributions of the 'basal resting Q_{O_2}', the 'tension Q_{O_2}' and the 'activity Q_{O_2}' to the total Q_{O_2} in a single experiment were determined as follows. The muscle was first placed in the chamber without tension and the 'basal resting Q_{O_2}' was determined. The tension of the resting muscle was then brought to the desired level and the 'tension Q_{O_2}' was determined. Finally, the muscle at this tension was stimulated at a rate of 60/min, and the 'activity Q_{O_2}' was determined.

At a stimulation frequency of 60/min, the length and the total oxygen consumption of muscles were functions of the resting tension. The relationship between the Q_{O_2} and the length in a typical experiment is shown in Fig. 9. Initially, the total oxygen consumption increased progressively as the resting tension increased, reached a maximum at approximately 10 g/mm² of resting tension and then declined slightly at higher tensions. This slight decrease in the total Q_{O_2} at higher tensions was mainly due to the decrease in 'activity Q_{O_2}' at these tensions. On the other hand, the length of the muscle gradually increased as the tension was increased up to 16 g/mm², beyond which further increase did not change the length significantly. Thus, both the total oxygen consumption and muscle length

increased as the testing tension was increased up to 10 g/mm²; however, beyond this tension the former declined despite the continued increase of the latter. The proportions contributed by resting, tension and activity Q_{O_2} to the total Q_{O_2} of this muscle are also shown in this figure. The total oxygen consumption at three different testing tensions was analysed into various components, and the results are summarized in Table 5. It is seen that the proportions these components contribute to the total oxygen consumption vary with resting tensions.

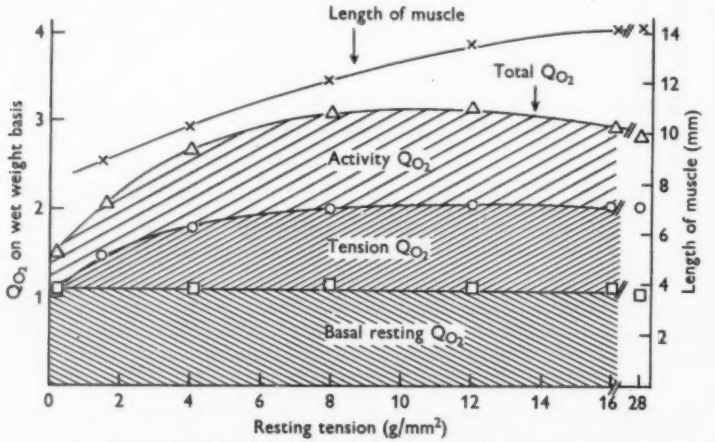

Fig. 9. The effect of resting tension on the length and oxygen consumption of the papillary muscle. Muscle diameter, 0·4 mm; wet weight 5 mg.

TABLE 5. Effects of resting tension on total oxygen consumption

No. of expts.	Resting tension (g/mm²)	Total Q_{O_2} ± S.E.	'Basal resting Q_{O_2}'* ± S.E.	'Tension Q_{O_2}'* ± S.E.	'Activity Q_{O_2}'* ± S.E.
5	1·2	2·2 ± 0·21	54 ± 7·6	16 ± 4·2	28 ± 5·5
6	6	2·7 ± 0·31	38 ± 7·1	29 ± 6·2	34 ± 6·2
9	12	3·35 ± 0·29	34 ± 8·8	32 ± 7·7	33 ± 8·8

* Expressed as % of total Q_{O_2}.

DISCUSSION

The technique described in this paper for the measurement of oxygen tension has been found to be highly satisfactory for the quantitative study of the oxygen uptake of isolated papillary muscles. As discussed in a previous paper (Lee, 1953) the thickness of the muscles used was well within the limit necessary for the adequate diffusion of oxygen throughout. In the resting state without tension, the Q_{O_2} of muscles, on a wet weight basis, was found to be 1·16. An increase in the tension applied to resting

muscle was accompanied by a gradual increase in the Q_{O_2}. The maximum increase in the Q_{O_2} due to an increase in the tension on the muscle was reached when the tension approximated 13 g/mm^2. The average maximum Q_{O_2} due to tension was about 90 % of the 'basal resting Q_{O_2}'. This would indicate the importance of the influence of resting tension on the oxygen uptake of heart muscle.

Contraction, initiated by electrical stimulation, was found to increase the Q_{O_2} of the muscle as shown in Fig. 4. In this figure both the transition from the steady resting respiration to the steady active respiration following the initiation of contraction and the reverse transition following the cessation of contraction can be expressed by exponential functions of time. This temporal relationship characterizing the increase and decrease of respiration following the initiation and cessation of the contraction may describe the characteristics of the sequence of processes linking contraction with aerobic metabolism.

When muscle was contracting isometrically under a constant resting tension, both 'activity Q_{O_2}' and the contractile tension were found to vary with the frequency of contraction. Over the range of lower frequencies of contraction, both 'activity Q_{O_2}' and contractile tension were increased as the rate of stimulation was increased. The maximal contractile tension under constant resting tension was reached when the frequency of contraction was in the range of 60–90/min. When the frequency was further increased beyond this range, however, the 'activity Q_{O_2}' increased on one hand and the contractile tension decreased on the other. The maximal 'activity Q_{O_2}' under a constant resting tension was obtained when the muscle contracted at approximately 110/min, and reached about 90 % of the 'basal resting Q_{O_2}'. When the 'activity Q_{O_2}' is divided by the frequency of contraction per hour and this ratio is assumed to be the 'activity oxygen consumption per contraction', it is seen in Table 3 that an inverse relationship holds between the frequency of contraction and the 'activity oxygen consumption per contraction'.

The relationship between the 'activity Q_{O_2}' and contractile tension under a constant resting tension is expressed in terms of a hypothetical mechanical efficiency for various frequencies in Fig. 6. As was described above, the index of mechanical efficiency was calculated by the ratio, contractile tension : 'activity oxygen consumption per contraction'. Although the assumption that the oxygen consumption is a constant index of the energy production of the heart may be inaccurate *in vivo*, owing to factors such as changing respiratory quotient, this assumption is considered to be reasonably valid in this study, since glucose was the only substrate available in the medium and hearts of warm-blooded animals contract on an oxygen debt only for a brief period, if at all (Evans, 1939; Gollwitzer-

Meier, 1939). Thus the above ratio may reasonably be assumed to be an indicator of the mechanical efficiency. If so, Fig. 6 suggests that, when a papillary muscle is contracting under a constant tension, the highest mechanical efficiency is observed at stimulation frequencies of 60–100/min, and the efficiency decreases at both higher and lower frequencies.

When the muscle is contracting isometrically at a constant frequency (60/min), both the 'activity Q_{O_2}' and the contractile tension vary with the resting tension (Fig. 7). Over a range of lower tensions both the 'activity Q_{O_2}' and the contractile tension increase as the resting tension is increased. The maximum contractile tension is obtained when the muscle is contracting under a resting tension of 4–8 g/mm². When the resting tension is increased further, the 'activity Q_{O_2}' increases on one hand and the contractile tension decreases on the other. The maximum 'activity Q_{O_2}' is observed when the muscle is contracting under a resting tension of 10–14 g/mm². Thus it is seen that, when a muscle is contracting at a constant rate, the maximal oxygen consumption is obtained at a greater resting tension than that at which the maximal contractile tension is obtained. Previously Evans & Hill (1914) found the same relationship between the heat production and the contractile tension during isometric contraction of *frog* skeletal muscle at different resting tensions. If the oxygen consumption is considered to be a reasonable index of heat production in the present experiment, these results would indicate that the relationship between the contractile tension, heat production and the initial tension found in frog skeletal muscle also applies to a mammalian cardiac muscle.

When the hypothetical index for the mechanical efficiency is calculated as described previously for different resting tensions, it is seen (Fig. 8) that the mechanical efficiency rises sharply over the range 0–3 g/mm². The change in mechanical efficiency of heart muscle under varying diastolic volume has been the subject of many studies (Starling & Visscher, 1927; Moe & Visscher, 1939; Katz, Jochim, Lindner & Landowne, 1941; Wise, Meyer, Katz, Lendrum & Jochim, 1946). Starling & Visscher (1927) found that as the heart goes into failure while the diastolic volume increases, there is a decrease in the mechanical efficiency. The validity of their conclusion was questioned because of inherent difficulties involved with whole-heart preparation in the measurement of the true mechanical efficiency (Katz, 1955). The major difficulty was that the work done by the heart could not be correlated with the oxygen uptake due to activity only and total oxygen uptake had to be used in their calculation. In the present study the index of mechanical efficiency was calculated by using the oxygen consumption due to activity only, and the interference by other factors was completely eliminated. Under these experimental con-

ditions, the results shown in Fig. 8 indicate that the mechanical efficiency of the heart muscle starts to decline when the initial resting tension increases beyond 4 g/mm². Since the length of muscle continues to increase as the resting tension is increased beyond 4 g/mm² (Fig. 9), the present conclusions are in accord with the finding of Starling & Visscher (1927) that an increase in the diastolic volume beyond certain limits is accompanied by a decrease in the mechanical efficiency. It is interesting to consider the mechanical efficiency of the cat papillary muscle under varying resting tensions and frequencies of contraction. When the frequency is the only variant the muscle has the highest mechanical efficiency over the range of 60–100/min. When the resting tension is the only variant the muscle has the highest mechanical efficiency over the range of 2–4 g/mm² of the resting tension. Thus it appears that a muscle contracting at a rate of 60–90/min under a resting tension of 2–4 g/mm² has the highest mechanical efficiency.

When muscles contract at a rate of 60 beats/min, analysis of the total oxygen consumption (Fig. 9 and Table 5) shows that the 'activity Q_{O_2}' and 'tension Q_{O_2}' occupy a greater proportion of the total oxygen consumption at higher resting tensions and the 'basal resting Q_{O_2}' occupies a greater proportion at lower resting tensions. The maximal total oxygen uptake of muscle contracting at a rate of 60/min is observed when muscles contract under a resting tension of 12 g/mm² or more. Under these conditions the 'tension Q_{O_2}' and the 'activity Q_{O_2}' are approximately 80 and 89% of 'basal resting Q_{O_2}'. It is interesting to note that at the maximal total oxygen uptake of muscle, the activity, the tension and the resting state each accounts for approximately one third of the total oxygen uptake. The total Q_{O_2} at this time would be approximately 270% of 'basal resting Q_{O_2}' corresponding to 3·14. On the other hand, under conditions giving the maximal efficiency, namely, with a resting tension of 2–4 g/mm² and a frequency stimulation of 60 min, the 'tension Q_{O_2}' and 'activity Q_{O_2}' would amount to approximately 50 and 70% of the 'basal resting Q_{O_2}', respectively.

SUMMARY

1. A system is described whereby, by using polarographic principles for the determination of oxygen concentration and an RCA transducer for recording contractions, the simultaneous measurement of oxygen consumption and of contraction of the electrically stimulated cat papillary muscle is achieved. With this system the influence of tension and frequency of stimulation on the oxygen consumption and mechanical efficiency of the papillary muscle has been investigated.

2. An increase in the resting tension produced an increase in the oxygen consumption of resting muscle. The maximal increase in the Q_{O_2} of the resting muscle was obtained when the resting tension was 12 g/mm² or greater.

3. Electrical stimulation of the muscle produced an increase in the Q_{O_2}. This 'activity Q_{O_2}' increased as the frequency of stimulation increased and reached a maximum at a frequency of 110/min or greater.

4. The papillary muscle contracting under a constant tension has its greatest mechanical efficiency at a stimulation frequency of approximately 90/min. On the other hand, with a constant frequency of stimulation the greatest mechanical efficiency was attained under a resting tension of about 3 g/mm².

5. The extent of contribution of each of the following—the 'resting Q_{O_2}', 'tension Q_{O_2}' and 'activity Q_{O_2}'—to the total Q_{O_2} of the contracting muscle was found to be influenced by the initial resting tension of the muscle.

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THE FATE OF SUBSTANCES INJECTED INTO THE ANTERIOR CHAMBER OF THE EYE

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When a substance is injected into the anterior chamber of the eye there are, theoretically at any rate, two main routes by which it may escape into the blood stream. These are: (*a*) by a bulk flow into Schlemm's canal, and (*b*) by diffusion into the iris whence it will be carried away in the capillary circulation of this tissue. If a given substance could be shown to leave exclusively by the bulk flow mechanism, then we should have the possibility of measuring the rate at which this flow occurred, since it would simply be related to the rate at which the concentration of the substance fell after being injected into the anterior chamber. In the present study, therefore, an attempt has been made to establish the relative extents to which several substances leave the anterior chamber by these two pathways. The main difficulty that has so far frustrated quantitative studies of this sort has been the upset in the normal physiology of the eye that results from the insertion of a hypodermic needle into the anterior chamber and its subsequent removal. The abrupt changes in intraocular pressure caused by the injection, and the subsequent unavoidable leakage of aqueous humour on removal of the needle, lead to a break-down of the blood-aqueous barrier, with the passage of plasma proteins into the aqueous humour. This break-down of the barrier would obviously invalidate any conclusions as to the normal pathway of escape of the injected substance, whilst the leakage would forbid any attempt at a quantitative treatment. In the present study this difficulty has been overcome by adopting a continuous injection procedure, the rate of injection of the solution being so small as not measurably to alter the intraocular pressure. The cat was used almost exclusively because the large volume of aqueous humour in its eye permitted the analysis of several different substances in duplicate samples. For comparative purposes a few experiments were carried out on the rabbit; rodents differ from the cat in having a very incomplete endothelium on the anterior surface of the iris, so that one might expect a more

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ready escape of material into the iris of the rabbit. In fact, however, as we shall see, escape by way of the iris would appear to have been negligible in both species. No record of room temperature—which might have affected convection, and therefore mixing in the anterior chamber—was kept. The experiments were carried out in the months of August and September 1957–59, so that there was a fairly wide spread.

THEORETICAL

For a substance that leaves the anterior chamber only by flow, the rate of flow of aqueous humour can be computed from the experimentally determined parameters by the following highly simplified treatment.

If S is the amount of the injected material in the aqueous humour at any time, t, we have

$$dS/dt = xC_{Inj} - (x+f)C_{Aq}, \quad (1)$$

where x is the rate of injection,
f is the rate of flow (i.e. rate of production of aqueous humour),
C_{Inj} is the concentration of the substance in the injection fluid,
and C_{Aq} is the concentration in the aqueous.

The assumption at the basis of this equation is that mixing of the injected material throughout the fluid in the anterior chamber is very rapid.

Since the concentration of the substance in the aqueous humour, C_{Aq}, is equal to S/V, where V is the volume of the aqueous humour in the anterior chamber, we have

$$dC_{Aq}/dt = \frac{x}{V}C_{Inj} - \frac{v+f}{V}C_{Aq}. \quad (2)$$

This, on integration, and applying the limiting condition: $t = 0$, $C_{Aq} = 0$, gives:

$$\ln \frac{C'_{Inj}}{C'_{Inj} - C_{Aq}} = \frac{x+f}{V}t, \quad (3)$$

where C'_{Inj} has been written for $x/(x+f).C_{Inj}$ and V has been taken as effectively constant.

With x, C_{Aq}, t and V all known, f may be calculated by successive approximations. It will be seen later, however, that the principal assumption, that there is rapid mixing of the injected substance, does not hold; moreover, a significant fraction of the total loss of material from the anterior chamber takes place into the cornea and sclera. The equation is of use, nevertheless, in showing roughly how the measured loss of injected material will be affected by variations in such parameters as the rate of flow of aqueous humour, and the volume of aqueous humour in the anterior chamber. Thus, calculation shows that the effects of variations in the rate

of flow, from 5 to 30 μl./min, on the observed loss of injected material 1 hr after the beginning of the injection, will be as follows:

Rate of flow (μl./min)	5	10	15	20	25	30
Loss (% of injected)	16	27·5	37	43·5	50	55

assuming an injection rate of 1·5 μl./min and a volume of aqueous humour 1000 μl. Thus, a threefold increase in rate of flow should only double the loss, expressed as a percentage of the amount injected.

Again, for a rate of injection of 1·5 μl./min, a rate of flow of 15 μl./min, and an injection period of 1 hr, the expected effects of variations in the volume of aqueous humour in the anterior chamber are as follows:

Volume (μl.)	500	750	1000	1250
Loss (% of injected)	56·5	44·5	37	31·5

METHODS

As already stated, cats were used principally; a few experiments were carried out on rabbits for comparative purposes. All experiments were carried out under general (pentobarbitone) anaesthesia. The experimental procedure is illustrated diagrammatically in Fig. 1. The piston of an Agla 1 ml. syringe, *S*, is driven at a constant rate by the infusion machine described by Davson & Purvis (1952); fluid containing the substance to be injected passes through the graduated capillary tube, *C*, and thence into the eye by way of polythene tubing and a specially sharpened gauge 18 hypodermic needle, *N*. The two-way junction, *J*,

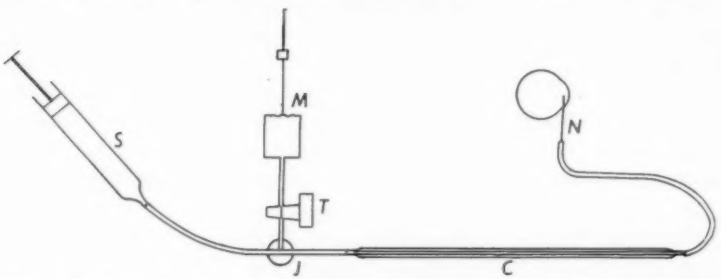

Fig. 1. Illustrates the experimental arrangement. Fluid is driven from the syringe, *S*, through the capillary tube, *C*, and needle, *N*, into the eye. The manometer, *M*, records the intraocular pressure.

and the tap, *T*, join the injection system to the manometer, *M*, which permits the recording of the intraocular pressure throughout the experiment. The manometer is that described recently by Davson & Purvis (1959). Movement of a bubble of air, introduced into the capillary tube, gives an exact measure of the amount of fluid injected. After cocainizing the eye for at least 15 min, and with the tap *T* closed, the needle was inserted into the anterior chamber in such a way that it penetrated the cornea at the limbus and rested with its opening close to the pole of the cornea. The tap was then opened, and the intraocular pressure recorded for several minutes. The injection machine was then started and kept going at a constant speed for the required time, which varied from 60 to 120 min. At the end of the period the machine was stopped, the tap *T* turned, the movement of the bubble

noted, and the whole of the aqueous humour removed by inserting a second needle, connected to a weighed 2 ml. syringe, into the anterior chamber. The syringe was weighed again to give the weight of aqueous humour. Chemical and isotopic analysis of the aqueous humour, and of a dilution of the injection solution, permitted the calculation of the amounts of the various substances that had escaped from the anterior chamber.

The analytical methods were the same as those employed in earlier studies (Davson, 1955).

RESULTS

Injection into the dead eye. A useful test of the reliability of the estimates of the loss of injected material is given by injecting it into the dead eye of the freshly killed animal. Under these conditions the loss to the blood will be very small, because of the absence of circulation of both aqueous humour and blood. After insertion of the needle into the eye the animal was killed with an overdose of pentobarbitone. Injection of a solution of ^{24}NaCl was maintained at about 1·5 μl./min for 1 hr, and after removing the aqueous humour the eye was removed and dissolved in concentrated HNO_3. A typical balance sheet was as follows:

Counts injected		4350
Counts recovered: Aqueous humour	3250	
Rest of eye	1050	
Total		4300

Relative rates of loss of ^{24}Na, PAH *and sucrose.* When injected into the blood, *p*-aminohippurate (PAH) and sucrose enter the aqueous humour very slowly and at about the same rate (Davson, 1955); ^{24}Na enters very much more rapidly. The mean results of eleven experiments, in which these three substances were injected together into the aqueous humour for periods varying from 60 to 120 min, were as follows:

	^{24}Na	PAH	Sucrose
Loss (% of injected)	59·5	61·5	61·5

The mean difference in the percentage losses of ^{24}Na and PAH was $2 \pm 0{\cdot}8$ (S.E. of mean difference), the loss of PAH being actually the greater; the mean difference in the percentage losses of ^{24}Na and sucrose was $2 \pm 1{\cdot}3$, the loss of sucrose being the greater. These differences are sufficiently close to the standard errors to be insignificant. During the course of this work the losses of PAH and ^{24}Na were compared in a great many experiments under different conditions; the mean difference, percentage loss of ^{24}Na minus percentage loss of PAH for thirty-eight of these experiments, was $3{\cdot}0 \pm 0{\cdot}8$, so that it would seem that the loss of PAH may be just significantly less than that of ^{24}Na.

Escape of PAH when the plasma concentration is high. The finding that ^{24}Na, PAH and sucrose all leave the eye at about the same rate strongly

suggests that they are all leaving by bulk flow through a non-selective channel, for example, by flow into Schlemm's canal. If this were indeed the mechanism of egress, we should not expect the rate to be materially influenced by the concentration of the substance in the blood. This may be put to a test by employing PAH as the injected substance, since its penetration into the aqueous humour is slow enough not to obscure the losses due to bulk flow out of the eye. In the following experiments the concentration of PAH in the plasma was maintained higher than that attained in the aqueous humour by intraocular injection. Only one eye was injected, and at the end of 60 min both aqueous humours were removed and analysed. An approximate correction for the slow penetration of PAH into the injected eye was made by subtracting the amount found in the other eye from that found in the injected eye. (This correction will only be valid, of course, if the penetration of PAH is independent of the concentration in the aqueous humour, but since the concentration in the uninjected eye was only some 10 % of that in the injected eye, an error in this correction is not of much significance.) The injection fluid contained PAH, ^{24}Na and sucrose; as we have seen, these normally leave the eye at about the same rate, so any effect of the high concentration of PAH in the blood will be shown by a discrepancy in the losses of these three substances. The mean results of five experiments were as follows:

	^{24}Na	PAH	Sucrose
Loss (% of injected)	53·5	56·5	59

It is quite clear that the presence of PAH in the blood, which would have put a complete stop to any losses by simple diffusion, has had no significant effect on the escape from the aqueous humour.

Losses to the ocular tissues. The experiments on the dead eye showed that a significant proportion of the injected material remained in the eye after removal of the aqueous humour. In the living eye we may expect this proportion to be less, because the concentration in the aqueous humour will not build up so rapidly, but it may nevertheless represent a significant portion of the loss that has so far been treated as an escape from the eye into the blood. In the following experiments ^{24}Na or PAH was injected into the aqueous humour for 1 hr, and the eye was removed for analysis after withdrawal of the aqueous humour. The results of an experiment in which the various ocular tissues were analysed separately are shown in Table 1. It is quite clear that the amount of ^{24}Na entering the cornea and sclera represents a significant contribution to the total loss; thus, on the basis of the ^{24}Na recovered in the aqueous humour alone, the loss of injected ^{24}Na would be 64 %, whereas the true loss from the eye was less than this, namely 53 %. The mean results of four experiments gave an average apparent loss of 56·5 % and a corrected loss of 46 %. The mean of

six experiments in which PAH was used as the injected substance gave an average apparent loss of 42 % compared with a true loss of 32 %.

Escape of lipid-soluble substances. Ethyl alcohol injected intravenously penetrates the aqueous humour some five times as rapidly as ^{24}Na; this means that there is a significant diffusion of alcohol from the blood in the iris directly into the anterior chamber; perhaps the amount entering by this route is as much as three times that entering in the newly secreted aqueous humour. Propyl thiourea penetrates some two or three times as rapidly as ^{24}Na, so that with this substance, too, diffusion from the iris is a significant factor. (These figures apply to the rabbit, but comparative

TABLE 1. Recovery of intraocularly injected ^{24}Na from aqueous humour and the tissues of the eye

Counts injected			43,500
Counts recovered:	Cornea	2,830	
	Sclera	1,310	
	Uvea	590	
	Lens	10	
	Vitreous	10	
Total non-aqueous		4,750	
Aqueous humour		15,600	
Total recovery			20,350

studies on cat and rabbit have shown a great similarity in the quantitative aspects of the blood–aqueous barrier, so that there is little likelihood of error in applying results obtained on the one animal to the other.) We may therefore expect the losses of alcohol and propyl thiourea to be considerably greater than those of ^{24}Na and PAH. The mean results of ten experiments in which ^{24}Na, PAH and ethyl alcohol were injected together for 1 hr are as follows:

	^{24}Na	PAH	Ethyl alcohol
Loss (% of injected)	49	48	62·5

The mean difference in the percentage losses of ^{24}Na and ethyl alcohol was $13{\cdot}5 \pm 1{\cdot}5$ (S.E. of mean difference).

The loss of ethyl alcohol is surprisingly small, especially as experiments carried out in another connexion have shown that there is a considerable metabolic consumption of ethyl alcohol by the tissues of the eye. With propyl thiourea the loss is only a little greater than that of ^{24}Na, as the following results on fourteen eyes show:

	^{24}Na	Propyl thiourea
Loss (% of injected)	50	53·5

The mean difference in the percentage losses of ^{24}Na and propyl thiourea was $3{\cdot}5 \pm 0{\cdot}7$.

Effects of atropine and eserine. In experiments on eight animals, one eye was treated with atropine and the other with eserine before starting the injection. Because in any animal there is a tendency for the loss in the second eye to be less than that in the first (see below), in half the animals the first eye was treated with atropine and in the other half the first eye was treated with eserine. The injection solution contained ^{24}Na and PAH in all cases; in six experiments it also contained ethyl alcohol, and in four experiments propyl thiourea. Mean values for the losses, expressed as percentages of the amount injected, were as follows:

^{24}Na		PAH		Ethyl alcohol		Propyl thiourea	
Atr.	Eserine	Atr.	Eserine	Atr.	Eserine	Atr.	Eserine
37·5	52·5	34·5	47·5	51	63	47·5	55

In every case the loss was greater in the eserinized eye, so that no statistical analysis is necessary to prove the significance of the difference caused by the two drugs. It is of interest to see, however, whether both drugs have an effect, or whether the difference is entirely due to one or the other. The mean percentage loss of ^{24}Na from twenty-seven normal eyes was $48{\cdot}5 \pm 2$ (S.E. of mean), whilst the value for the eight atropinized eyes was $37{\cdot}5 \pm 2$ and that for the eserinized eyes $52{\cdot}5 \pm 4$. Thus the difference between the atropinized and eserinized eyes is due mainly to the reduction in loss caused by atropine. This is not surprising, since in most experiments on normal animals the pupil was strongly constricted during the course of the injection.

Effect of Diamox (acetazoleamide). It has been claimed that this drug decreases the rate of flow of aqueous humour by some 65 % (Becker, 1955), although the validity of this claim has been questioned (Davson & Luck, 1957). As is shown in the Theoretical section, a decrease in rate of flow from, say, 15 to 5 μl./min should be manifest as a fall of about 50 % in the loss of injected material occurring in 1 hr. Such a large effect should be detected quite easily by the present methods. Because there is a tendency, in any given animal, for the second eye to give a rather slower rate of escape than the first eye, a direct comparison of the two eyes—one before and one after giving Diamox—was not made; instead, Diamox-treated animals (300–400 mg intravenously) were compared with normal controls, 'first' and 'second' eyes being kept separate. The losses of ^{24}Na in 1 hr, expressed as percentages of that injected, were as follows:

	First eyes (10)	Second eyes (17)
Control	$49 \pm 2{\cdot}5$	$47{\cdot}5 \pm 3$
Diamox	45 ± 3	$44{\cdot}5 \pm 2$

In both cases there was a fall in the loss of ^{24}Na in the Diamox-treated eyes.

Mixing of injected material in aqueous humour. In these experiments the eye was injected for a definite period (1–2 hr) and the aqueous humour was removed in samples of 0·2 ml. in rapid succession, the needle used for the withdrawal being connected to a two-way tap that allowed the sample to be first drawn into a length of polythene tube and then rapidly ejected into a sample tube. The needle was held so that the earliest samples probably came from the fluid nearest the cornea. The results of an experiment, in which ^{24}Na and PAH were injected, are as follows, where the concentrations in the aqueous humour are expressed as percentages of that in the first sample:

	^{24}Na	PAH
(i)	100	100
(ii)	92	92
(iii)	73·5	69
(iv)	57·5	54
(v)	46	46

Rate of penetration of ^{24}Na from the blood. When ^{24}Na is injected into the blood continuously, so as to maintain a constant plasma concentration, C_{Pl}, the concentration in the aqueous humour, C_{Aq}, rises approximately in accordance with the simple equation

$$dC_{Aq}/dt = k_{in}C_{Pl} - k_{out}C_{Aq}.$$

If the ^{24}Na entering the aqueous humour passed in entirely with the newly formed aqueous humour, with the same specific activity as that in the plasma, k_{out} would be a measure of the rate of flow. If there were some diffusion of ^{24}Na from the iris, directly into the anterior chamber, k_{out} would be greater than the rate of flow; it may therefore be used as an approximate upper limit to the rate of flow. It is possible that the variations in rate of loss of injected material that occurred from one animal to the next were due, in part, to variations in the rate of flow of aqueous humour. If this were true, there would be a correlation between loss of injected material and k_{out}. In five animals k_{out} was measured with one eye whilst the other eye was used to measure the loss of injected ^{24}Na. No correlation was found; the mean value of k_{out} was 0·135/min compared with 0·014/min found in an earlier study (Davson, Duke-Elder, Maurice, Ross & Woodin, 1949). The rate of flow of aqueous humour would have a value of 1·3–1·4 % of the total volume per minute were k_{out} indeed a measure of rate of flow.

Intraocular pressure. If the escape of fluid into Schlemm's canal and the blood vascular system may be treated as a simple mechanical flow resulting from a difference of pressure in the aqueous humour (P_{Aq}) and in the episcleral veins (P_V), the flow will be given by:

$$\text{flow} = (P_{Aq} - P_V)/R,$$

where R is a term indicating the frictional resistance. According to Seidel (1923) the value of P_V is of the order of 10 mm Hg, so with an average intraocular pressure, P_{Aq}, of say 20 mm Hg the head of pressure driving fluid out of the eye is about 10 mm Hg. If the normal rate of flow is of the order of 15 μl./min (Bárány, 1955) an increase in this, due to injection at the rate of 1·5 μl./min, should only increase $(P_{Aq} - P_V)$ by 10%, i.e. by 1 mm Hg. In fact, in most of the experiments carried out in this work the injection had no observable effect on the intraocular pressure; in most of

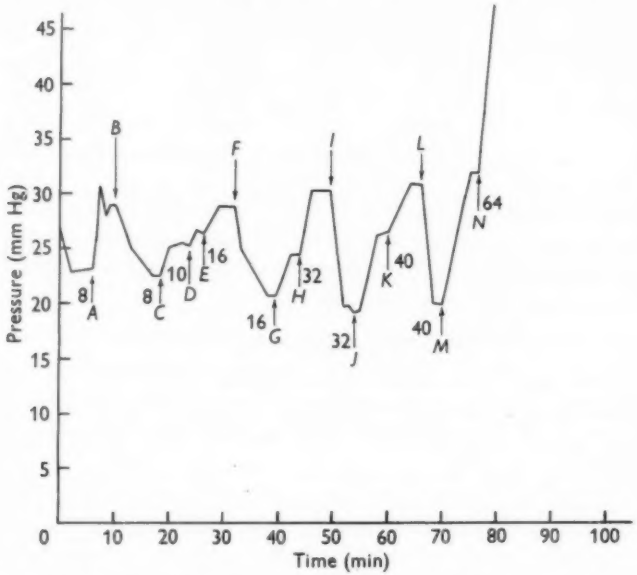

Fig. 2. Effects of injection of saline, at different rates, on the intraocular pressure. Upward arrows indicate beginning of injection; downward arrows indicate cessation of injection; numbers beside arrows indicate rates of injection in μl./min.

the experiments the pressure gradually fell, possibly because of the sustained anaesthesia. In the course of this work, however, the rate of injection was occasionally increased to very much greater values than 1·5 μl./min, but it was often found that the rise of intraocular pressure, if it occurred, was only transient, and there seemed little doubt that there was some adaptation to an increased rate of flow. An example of this is shown in Fig. 2, where the intraocular pressure has been plotted against time. At A the injection began at a rate of 8 μl./min, and the pressure rose to just over 30 mm Hg. On stopping the injection, at B, the pressure fell to just below its original value. On starting the injection at the same rate, at C, the pressure rose, but now only to about 26 mm Hg, and the rate

could be increased to 10 (*D*) and 16 (*E*) μl./min without raising the intraocular pressure above 29 mm Hg. At *F* the injection was stopped and the pressure fell now to 21 mm Hg. On starting again at 16 μl./min the pressure now only rose to 24 mm Hg and the rate could be increased to 32 μl./min without raising the pressure significantly above 30 mm Hg, the peak pressure that had originally been caused by an injection rate of only 8 μl./min. At *I* the injection was stopped, and on re-starting it at the same rate (*J*) the pressure only rose to 26·5 mm Hg, and it required a rate of 40 μl./min to cause the intraocular pressure to rise above 30 mm Hg. At this rate of injection, which may well be some three times the normal rate of flow, bringing the total rate of flow to some four times its normal value, the limit to the power of the eye to adapt seems to have been reached, since increasing the rate still further (*N*) caused a large rise in pressure.

Experiments on the rabbit

The volume of aqueous humour in the rabbit's eye is only about 0·35 ml. compared with 1 ml. or more for the cat. The rate of injection was therefore reduced to approximately 0·5 μl./min. Under these conditions application of equation 3 indicates that the loss in 1 hr should be comparable with that found in the cat, provided that the rates of flow, measured as the fraction of the total volume of aqueous humour, are the same. Mean results on nine eyes were as follows:

	^{24}Na	PAH
Loss (%)	32	33

As with the cat, ^{24}Na and PAH leave the eye at about the same rate, the mean difference in percentage loss for the nine eyes studied being $1·2 \pm 0·9$ (S.E.). The loss from the aqueous humour is significantly less than that found with the cat; thus loss of ^{24}Na from the latter eye was $48·5 \pm 2$% compared with $32 \pm 3·5$% for the rabbit.

DISCUSSION

The most striking feature of the results described here is the approximate uniformity in the rates of escape of different substances from the anterior chamber, which is in marked contrast with the rates at which they enter from the blood. Thus the values of k_{in} for sucrose, ^{24}Na, propyl thiourea and ethyl alcohol are probably in the ratios 1:10:30:70, yet the rates of loss of sucrose and ^{24}Na are the same, and those of propyl thiourea and ethyl alcohol only slightly larger. This strongly suggests that, under the conditions of the experiments, these substances leave either exclusively (sucrose, PAH and ^{24}Na) or nearly exclusively (propyl thiourea and alcohol) by way of an unselective channel, which could be Schlemm's

canal, separated as it is from the aqueous humour only by a thin endothelial layer. Moreover, the fact that the rate of escape is apparently independent of the plasma concentration, at any rate so far as PAH is concerned, proves unequivocally that the escape is by a flow mechanism rather than by diffusion. Consequently, within the limits imposed by the deficiencies of the experimental technique, the clearance of, say, ^{24}Na can give a semi-quantitative measure of changes in the rate of flow of aqueous humour.

It is known that lipid-soluble substances, such as ethyl alcohol, pass into the aqueous humour in the anterior chamber by diffusion from the blood in the iris, so the problem arises as to why, when these materials are introduced directly into the aqueous humour, there is only an insignificant loss by this channel. There are probably two factors that account for this: First, it may well be that there is a current of fluid passing from the iris into the anterior chamber, so that diffusion 'up-stream' is considerably prejudiced. Secondly, the normal flow of freshly secreted aqueous humour through the pupil, and the thermal currents in the anterior chamber, probably tend to sweep the injected material away from the surface of the iris, so that it reaches the angle of the anterior chamber before being significantly exposed to this tissue. The analyses of successive samples of aqueous humour support this view, the concentration of material in the sample close to the iris being only about a half of that close to the cornea. The lack of dependence of the rate of clearance on the state of the iris is remarkable. This is shown by the effects of atropine and eserine: in the atropinized eye with a small area of iris exposed the clearance is, indeed, reduced but the effect is just about as great with PAH and ^{24}Na as with ethyl alcohol, whereas we should expect it to be much larger with the lipid-soluble substances.

That the rate of clearance from the eserinized eye, with its tightly constricted pupil, was greater than that from the atropinized eye may have been due to a reduced volume of the anterior chamber of the eserinized eye, caused, apparently, by a bulging forward of the iris. (Having no slit-lamp microscope we were unable to make an objective measurement of the depth of the anterior chamber to check this impression; we may note, however, that Armaly (1959) has observed a bulging forward of the lens and iris on stimulation of the parasympathetic supply to the eye in the orbit.) As was shown in the Theoretical section, a change in volume of aqueous humour in the anterior chamber from 1 to 0·75 ml. could increase the loss in 1 hr from 37 to 44·5 %, on the assumption of a rate of flow of 15 μl./min. This compares with an increase from 37 to 49 % observed with PAH, when the atropinized eye is compared with the eserinized eye.

The failure to detect any large effect of Diamox on the rate of loss of

injected material is further confirmation of the view put forward by Davson & Luck (1957), that the fall in intraocular pressure caused by this drug is not accompanied by any large change in rate of secretion of aqueous humour. The earlier experiments, which showed that Diamox had no detectable influence on the rate of turnover of ^{24}Na in the aqueous humour (Davson & Luck, 1957) or vitreous body (Davson, 1958), indicated that any decrease in the rate of flow could not have been greater than about 10%. An analysis of the present experiments has shown that a drop of 7 units in the percentage loss in 1 hr would have been significant: this would have corresponded to a decrease of about 25% in the rate of flow. The failure to observe a decrease of more than 4 units in the mean percentage loss indicates that it is highly unlikely that there was a reduction of rate of flow as great as 25%, and it seems safe to conclude that the effect of the drug on the intraocular pressure is due in part to a change in the physical factors affecting drainage.

Thus, in many instances the intraocular pressure, after administration of Diamox, fell well below the value usually assigned to the pressure in the episcleral veins; and since the rate of escape of injected material was barely affected it is obvious that the episcleral venous pressure must have been very low to permit flow at about normal rate. If the primary action of Diamox is an attack on the process of secretion of aqueous humour, we may envisage its action as an impairment of the ciliary body's power to produce fluid against an opposing pressure; this leads initially to a decrease in rate of flow which is partly compensated, however, by a reduction in episcleral venous pressure which permits flow to be re-established at a rate not greatly different from normal, but with a lowered intraocular pressure.

The slower rate of clearance of injected ^{24}Na and PAH which is found with the rabbit may well be due to a relatively slower rate of secretion of aqueous humour, expressed as a fraction of the total volume. Certainly the rates of turnover of ^{24}Na are markedly different; with the large rabbits used for these experiments, k_{out} would have been about 0·009/min (Davson, 1955), which compares with 0·0135/min for the cat.

The remarkable ability of the cat's eye to accommodate an influx of fluid without a very large rise in intraocular pressure (shown in Fig. 2) must have been the consequence of a change in the factors favouring drainage, rather than an effect of decreased secretion of aqueous humour; these factors could have been a decrease in P_V, the episcleral venous pressure, and/or a decrease in the frictional resistance, R. Thus, when the eye was injected at a rate of 40 μl./min the pressure reached was about the same as that reached at the beginning of the experiment with a rate of only 8 μl./min. Even if secretion had ceased altogether during the high rate of injection, this could not have compensated for the extra 32 μl./min

entering the eye, unless present estimates of the rate of flow are too small by a factor of two. We may note that Langham (1959) has described a lack of linearity in the response of the intraocular pressure of the rabbit to injections of saline into the anterior chamber at different rates. This could have been due either to a reduction in P_V and R or to a reduced rate of secretion of aqueous humour, but the former alternative seems altogether the more reasonable as it is difficult to believe that a process of secretion, that may generate an intraocular pressure of 80 mm Hg, when the drainage routes are blocked (Adamiuk, 1867), could be significantly impeded by the small rises in intraocular pressure induced by the injections illustrated in Fig. 2, for example.

In conclusion it may be noted that we have abstained from attempting a calculation, from equation 3, of the rate of flow of aqueous humour. This is because mixing in the anterior chamber of the cat is slow, so that the fluid does not approach anything like homogeneity of concentration; furthermore, about 20% of the injected material diffuses into compartments that do not participate in the flow, notably the cornea and sclera. It is worth noting, nevertheless, that the average loss of 48·5% found with ^{24}Na, which corresponds approximately with a loss by flow of 38·5% when allowance is made for loss to the ocular tissues, is consistent with a rate of flow of the order of 15–20 μl./min on the assumption that equation 3 is applicable.

SUMMARY

1. A technique has been described for continuous injection of a solution at a known rate into the anterior chamber of the eye, whilst recording the intraocular pressure.

2. When ^{24}Na, sucrose and *p*-aminohippurate (PAH) were injected together they were carried out of the anterior chamber at approximately the same rate; when lipid-soluble substances, such as propyl thiourea and ethyl alcohol, were included in the solution they left the eye only slightly more rapidly. It was concluded that under these conditions material injected into the eye was carried out almost entirely by flow into Schlemm's canal; the rate of clearance could therefore be used as an approximate measure of the rate of flow of aqueous humour.

3. The rate of escape of PAH was unaffected by the plasma concentration, proving that the escape was by a flow rather than a diffusion mechanism.

4. When atropinized and eserinized eyes were compared, clearance from the eserinized eye was always the more rapid, owing, it was considered, to the reduced volume of the anterior chamber that occurred when the pupil was tightly constricted.

5. Diamox had no significant effect on the rate of clearance of injected material.

6. The above studies were carried out on the cat. In a few experiments on the rabbit it was found that, as with the cat, the rates of loss of ^{24}Na and PAH were the same. Both rates were significantly less in the rabbit than in the cat and implied a slower rate of turnover of aqueous humour.

7. Under appropriate conditions a rate of injection equal to some three times the probable rate of secretion of aqueous humour could be accompanied by only a small rise in intraocular pressure. It was shown that this adaptation must have been due to an increased facility for outflow from the eye, rather than a decreased secretion of aqueous humour.

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